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(71) Applicant *(for all designated States except US)*: ACADEMISCH ZIEKENHUIS UTRECHT [NL/NL]; Heidelberglaan 100, NL-3584 CX Utrecht (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BULL, Laura, N. [US/US]; 2614 47th Avenue, San Francisco, CA 94116 (US). FREIMER, Nelson, B. [US/US]; 630 29th Street, San Francisco, CA 94131 (US). HOUWEN, Roderick, H., J. [NL/NL]; Bosuillaan 28, NL-3722 XN Bilthoven (NL). KNISELY, Alexander, S. [US/US]; 933 Beech Avenue, San Francisco, CA 15233 (US). PAWLIKOWSKA, Ludmila [US/US]; 1547 11th Avenue, San Francisco, CA 94122 (US). VAN EIJK, Michiel, J., T. [NL/NL]; Tolsteegplantsoen 43, NL-3523 AN Utrecht (NL).

(74) Agent: OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).

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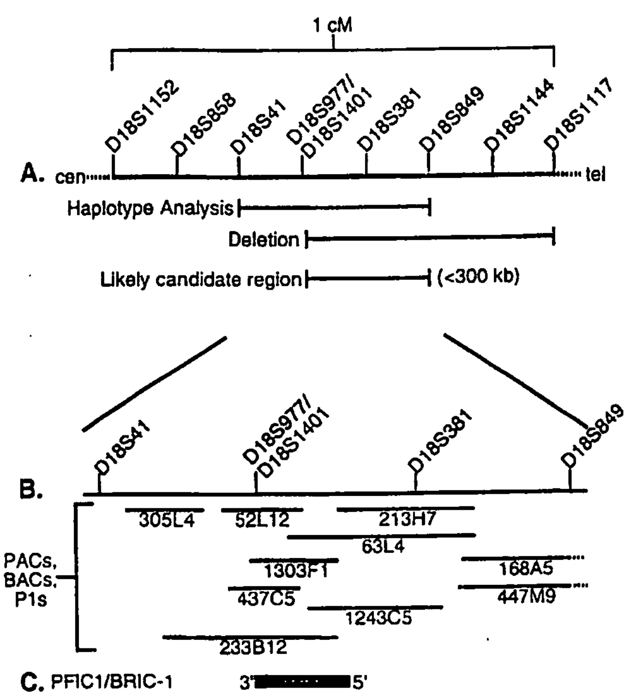
(57) Abstract

The invention relates to the field of gastroenteral disease in humans, more specifically to the field of liver disease, bile formation and bile acid secretion. In particular, it relates to gastroenteral disorders characterised by cholestasis, as for example seen with benign recurrent intrahepatic cholestasis and progressive familial intrahepatic cholestasis, or other disorders related to impaired bile flow or bile acid secretion, particularly in mammals. The invention provides an isolated and/or recombinant nucleic acid, or a functional fragment or homologue thereof, derived from a gene, which gene comprises a mutation in patients with benign recurrent intrahepatic cholestasis (BRIC) or progressive familial intrahepatic cholestasis (PFIC), said nucleic acid in humans having a sequence substantially identical to a nucleic acid sequence as shown in Fig. 5.



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NUCLEIC ACID CORRESPONDING TO MUTATION ASSOCIATED WITH CHOLESTASIS SYNDROMES

The invention relates to the fiel of gastroenteral disease in humans, more specifically to the field of liver disease, bile formation and bile acid secretion. In particular, it relates to gastroenteral disorders characterised by cholestasis, as for example seen with benign recurrent intrahepatic cholestasis and progressive familial intrahepatic cholestasis, or other disorders related to impaired bile flow or bile acid secretion, particularly in mammals.

Background of the Invention

Bile formation and bile acid secretion are necessary for the intestinal absorption of dietary fats and fat soluble vitamins, as well as for the elimination of cholesterol and various toxins. Cholestasis, or impaired bile flow, is one of the most common and devastating manifestations of hereditary and acquires liver diaese (Lidofsky and Scharschmidt, Gastointestinal and Liver Disease, M.Feldman, M.H.Sleisenger, and B.F.Scharschmidt, Eds. (WB Saunders Co, Philadelphia, ed. 6, 1997), pp. 220-234). It presents clinically with itchin, jaundice, and fat malabsorption (Lidofsky and Scharschmidt, Gastointestinal and Liver Disease, M.Feldman, M.H.Sleisenger, and B.F.Scharschmidt, Eds. (WB Saunders Co, Philadelphia, ed. 6, 1997), pp. 220-234). The molecular mechanisms of bile formation and of intraheptaci cholestasis remain poorly understood, in part because of the inaccessibility of the biliary canaliculi (a small intercellular space that lies between hepatocytes and through which bile is delivered from the hepatocytes to the bile ducts) and the intrahepatic biliary tree to direct physiological investigation.

[redacted]

Benign recurrent intrahepatic cholestatis BRIC (Summerskill syndrome) (Summerskill, Am.J.Med. 38:298 (1965); Bijleveld et al. Hepatology (:532 (1989): Bijleveld et al., Gastoenterology 97:427 (1989)) and progressive familial intrahepatic cholestasis type 1 PFICI (Byler disease) (Clayton et al., J.Pediatr. 67:1025 (1965); Linarelli et al., J.Pediatr. 81:484 (1972)) are of particular interest, because cholestasis is the predominant manifestation of these clinically distinct autosomal-recessive disorders (Straunieks et al., Am.J.Hum.Genet. 61:630 (1997); Bull et al., Hepatology 26:155 (1997)). BRIC is characterized by recurrent episodes of intrahepatic cholestasis, lasting days to months, which can begin in childhood or adulthood and resolve spontaneously, causing no detectable lasting liver damage (Summerskill, Am.J.Med. 38:298 (1965)). In contrast, PFICI is manifest in early infancy as cholestasis that may initially be episodic but progresses to malnutrition, growth retardation, and end-stage liver disease before adulthood (Clayton et al., J.Pediatr. 67:1025 (1965)). While these disorders differ greatly in severity, the share certain clinical and biochemical features (Jacquemin et al., Eur.J.Pediatr. 153:424 (1994)). Serum gamma glutamyl transpeptidase activity is low to normal in both BRIC (T.J.De Koning et al., Am.J.Med.Genet. 57:479 (1995) and PFICI (Bull et al., Hepatology 26:155 (1997)), contrasting with the elevated activity of this enzyme in most cholestatic disorders. Light microscopy in BRIC and early in the course of PFICI reveals only bland intracanalicular cholestatis (Summerskill, Am.J.Med. 38:298 (1965); Bull et al., Hepatology 26:155 (1997)). Substantially elevated concentrations of lithocholic acid, a strongly hepatotoxic bile acid, have been observed in BRIC and PFICI patients (Bijleveld et al. Hepatology 9:532 (1989);

Bijleveld et al., Gastoenterology 97:427 (1989); Linarelli et al., J.Pediatr. 81:484 (1972)), but not in patients with PFIC of unspecified sub-type (Jacquemin et al., Eur.J. Pediatr. 153:424 (1994)).

Based on these clinical findings it was earlier Clayton et al., J.Pediatr. 67:1025 (1965)) hypothesised that both disorders BRIC and PFIC1 are likely due to defective transport of bile acids across the canalicular membrane and, since currently identified proteins thas conduct active (in to out) transport across this membrane are ATP-binding cassette (ABC) proteins (Muller et al., Am.J.Physiol. 272, G1285 (1997)), it generally was assumed that said defective transport of bile acids across the canalicular membrane in BRIC and PFIC1 must likely be due to defects in said ABC protein genes.

Bile acids are synthesised in hepatocytes. They are secreted across the canalicular membrane into the biliary tract and thence into the small intestine. In the intestine they are absorbed and returned, through the portal circulation where thay are re-secreted into bile. Bile acid synthesis is in general not defective in BRIC or PFIC1, thus membrane malfunction or defetive canalicular transport in general results in liver injury through intrahepatic accumulation of bile acids, resulting in the above described clinical symptoms.

Therapy against above cholestatic disorders in general consists of dietary restrictions, successful drug treatment is in general not available, Occasionally, liver transplants are done in severe PFIC1 cases.

The invention provides an isolated and/or recombinant nucleid acid, or a functional fragment or homologue thereof, derived from a gene, which gene comprises a mutation in patients with benign recurrent intrahepatic cholestatis

(BRIC) or progressive familial intrahepatic cholestasis (PFIC), said nucleic acid in humans having a sequence substantially identical to a nucleic acid sequence as shown in Fig.5.

The invention provides for the first time insight in the molecular genetic background of cholestatic disorders such as BRIC and PFIC, especially in mammals, such as humans. Surprisingly, said molecular background is nog found in the overall absence of functioning ABC genes in patients suffering from said disorders, but is found in a BRIC/PFIC1 gene provided by the invention. Diverse mutations or alterations in said gene contribute to various forms of cholestasis that are known from these patients. Now that the molecular genetic background of cholestasis is provided by the invention, the invention provides means and methods to study said hereditary cholestases, for example in cells and/or experimental (transgenic) animals provided with a nucleic acid or fragment thereof as provided by the invention, thereby allowing the discovery or drugs that are beneficial to treatment of said disorders.

In a preferred embodiment, said nucleic acid (fragment) as provided by the invention is related to a gene belonging to the family of P-type ATPases. Said ATPase as provided by the invention is involved in the regulation of membrane activities in a multitude of cells throughout the body. Notably, said ATPase, when mutated or altered, is predominantly involved in gastoenteral disorers, such as thos of the (small) intestine, or those related to cholestasis.

The invention also provides a vector and/or cell comprising a nucleic acidaccording to the invention. Such a vector and/or cell is preferentially used to produce said cholestatin or functional (biologically active) fragments

thereof. Additionally, the invention provides a transgenic animal, such as a mouse, comprising a nucleic acid according to the invention. Such an animal, preferably comprising a mutated nucleic acid according to the invention which may also detectable with cholestasis patients, can advantageously be used to study said gastroenteral disorderd. Transgenic animals in general are abtainable via methods known to those skilled in the art.

The invention also provides a gene delivery vehicle comprising a nucleic acid or a vector according to the invention. Such a gene delivery vehicle is for example known from gene therapy wherein therapeutic genes are deliverd to tissues of interest and comprises for example a liposome, viral particle or vector. Animal, cell, vector, antibody, gene delivery vehicle, protein (fragment) or nucleic acid (fragment) as provided by the invention can also advantageously be used to detect or develop a drug for use in a treatment against membrane malfunction, for example in gastroenteral disease, such as intestinal disease or cholestasis. Known drug compounds can now be tested for relevant activity, drugs can for example be designed via combinatorial chemistry.

The invention also provides said an isolated protein (herein also called cholestatin), or functional fragments thereof, encoded by said gene for example a protein or polypeptide having an amino acid sequence substantially identical to the sequence as shown in figure 3. In a preferred embodiment, said protein or fragment is derived in a recombinant form from a nucleic acid (fragment) as provided by the invention.

Biologically active fragments of cholestatin, for example comprising agonistic or antagonistic cholestatin activity derived from said protein as provided by the

invention are also provided by the invention.

The invention also provides a pharmaceutical composition comprising a nucleic acid, or fragment thereof, or pharmaceutical composition comprising a protein, or fragment thereof, according to the invention. Such a pharmaceutical composition, or medicament comprising said nucleic acid or polypeptide, orfragments thereof, is useful in a method for treating for example cholestasis, or other disorders related to membrane malfunctioning, such as for example BRIC or PFIC, but also intestinal disorders related to membrane malfunction.

The invention also provides use of a nucleic acid as provided by the invention, or fragment thereof, a protein of polypeptide as provided by the invention, or fragment thereof, as provided by the invention in the preparation of a medicament for the treatment of cholestasis or other disorders related to membrane malfunctioning, such as for example BRIC or PFIC or intestinal disorders.

One aspect of the invention is a method of treating cholestasis in a patient, the method comprising administering to the patient a therapeutic dose of a polypeptide comprising a polypeptide having an amino acid sequence substantially identical to the sequence as shown in Figure 3. In some embodiments the cholestasis is benign recurrent intrahepatic cholestasis (BRIC) or progressive familial intrahepatic cholestasis type 1 (PFIC1).

A further aspect of the invention is a method of treating cholestasis in a patient, the method comprising administering to the patient a therapeutic dose of a polynucleotide comprising a polynucleotide having a nucleotide sequente substantially identical to the sequence as shown in Figure 3. In some embodiments the cholestasis is benign recurrent intrahepatic cholestasis (BRIC) or progres-

sive familial intrahepatic cholestasis type 1 (PFIC1).

A further aspect of the invention is a composition comprising an isolated polynucleotide comprising a nucelic acid sequence substantially identical to the sequence of Figure 5.

A further aspect of the invention is a composition comprising an isolated polypeptide comprising an amino acid sequence substnatially identical to the sequence of Figure 3.

A further aspect of the invention is a method for detecting a mutation in the PFIC1/BRIC gene, the method comprising amplifying a PFIC1/BRIC gene sequence in a nucleotide sample from a patient using a primer substantially identical to a nucleotide sequence of at least 8 consecutive nucleotides of Figure 5. In an embodiment, the mutation results in cholestasis in a patient. In an embodiment, the mutation is G308V, G892R, L288S, 2097+2(T-C), I1661T, 795delGNR, 2286-4, -3(CT-AA).

A further aspect of the invention is a method for diagnosing the disease cholestasis in a patient, the method comprising detecting a mutation in the PFIC1/BRIC gene, wherein the presence of the mutation indicates a likelihood of having cholestasis. In an embodiment, the mutation is G308V, G892R, L288S, 2097+2(T-C), I1661T, 795delGNR, 2286-4, -3(CT-AA).

Brief Description of the Figures

Figure 1 comprises panels 1A, 1B and 1C and graphically depicts the positional cloning of the PFIC1/BRIC gene.

1A. Genetic map of the one cM region containing the PFIC1/BRIC gene, indicating the microsatellite markers used for genotyping studies. The location of the candidate region for this gene, based on haplotype and deletion analyses, is shown.

1B. Physical map of the region (defined by markers D18S41 and D18S49) to which the PFIC1/BRIC gene was localized by haplotype analysis.

1C. The position of the candidate cDNA (PFIC1/BRIC-1) is shown in relation to the physical map.

Figure 2 depicts a Northern blot showing expression pattern of a candidate (partial cDNA) fot the PFIC1/BRIC gene. In panel A of the Northern blot ^{32}P-labelled 1.6 KB cNDA probe was hybridized to multiple tissue Northern blots (Clontech) containing 1-6 µg of poly(A)$^{+}$RNA in each lane. The membranes were washed with 0.1 X standard saline citrate (SSC) and 0.1% SDS, then exposed to x-ray film. Panel B of the Northern blot depicts hybridization of these same blots with a beta actin probe (Clontech).

Figure 3 depicts the predicted amino acid sequence encoded by PFIC1/BRIC-1 cDNA. Boxed sequences are P-type ATPase signature domains; bold sequences within the boxes are characteristic of members of a newly defines family of P-type ATPases (Xiaojing et al., Science 272:1495 (1996)). Putative transmembrane domains (predicted wit theaid of a Kyte-Doolittle hydrophobicity plot (Kyte et al., J.Mol.Biol. 157:105 (1982)) are underlined.

Figure 4 graphically depicts the location of PFIC1 and BRIC mutations in the coding sequence of PFIC1/BRIC-1. Vertical black bars represent the putative transmembrane domains.

Figure 5 depicts the cDNA nucleotide sequence of the PFIC1/BRIC-1 gene. The initiation and termination codons are underlined.

Specific Embodiments of the Invention

Cholestasis, or impaired bile flow, is an important but poorly understood manifestation of liver desease. Two distinct forms of inherited cholestasis, benign recurrent

intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis type 1 (PFIC1), were previously mapped to 18q21. Haplotype analysis narrowed the candidate region for both diseases to the samen interval of less than one centiMorgan, in which we identified a gene mutated in BRIC and PFIC1 patients. This gene is the first identified human member of a recently described family of P-type ATPases; ATP-dependent aminophospholipid transport is the previously described function of members of this family. This gene is expressed in several epithelial tissues and, surprisingly, more strongly in small intestine than in liver. Its product, cholestatin, is likely essential in entero-hepatic circulation of bile acids; further characteriazion of this protein will facilitate understanding of normale bile formation and cholestasis.

As disclosed herin, both BRIC and PFIC1 are caused by mutations in a single gene, the PFIC1/BRIC gene Unexpectedly, the initial characteriazion of this gene and its expression pattern, together with clinical and biochemical evidence, implicate abnormal function of the small intestine, as well as of the liver, in the pathogenesis of these disorders.

Thus, one embodiment of the invention is a purified polypeptide comprising the sequence of Figure 3. This polypeptide, cholestatin, can be used therapeutically to treat patients having or at risk of having cholestasis. In a further embodiment, the invention provides an isolated polynucleotide comprising the sequence of Figure 5. This polynucleotide can be used in vitro to produce therapeutic polypeptides for the treatment of cholestasis. The polynucleotide can also be used in both in vivo and in vitro gene therapy. In some embodiments of the invention, polynucleotide probes and/or primers are provided to amplify

sequences in the genome of a host having homology to the sequence of Figure 5. Such probes are useful in diagnostic assays for the presence of mutations, polymorphisms, or other sequence variants in the PFIC1/BRIC gene.

The term "nucleic acids", as used herin, refers to either DNA of RNA. "Nucleic acid sequence" or "polynucleotide sequence" refers to a single- of double-stranded polymer of deoxyribonucleotide of ribonucleotide bases read from the 5' to the 3' end, and include both self-replicating plasmids infectious polymers of DNA of RNA and nonfunctional DNA or RNA.

"Nucleic acid probes" may be DNA of RNA fragments. DNA fragments can be prepared, for example, by digesting plasmid DNA, or by use of PCR, or synthesized, for example, by the phosphoramidite method described by Beaucage et al., Tetrahedron Lett. 22:1859-1862 (1981), or by the triester method according to Matteucci, et al., J.Am.Chem.Soc. 103:3181 (1981), both incorporated herein by reference. A double stranded fragment may then be obtained, if desired, by annealing the chemically synthesized single strands together under appropriate conditons or by synthesizing the complementary strand using DNA polymerase with an appropriate primer sequence. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well in situations where the target is a double-stranded nucleic acid.

The phrase "selectively hybridizing to" refers to a nucleic acid probe that hybridizes, duplexes orbinds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation oftotal cellular DNA or RNA. "Complementary" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectively

hybridize to a nucleic acid probe. Proper annealing conditions depend, for example, upon a probe's length, base composition, and the number of mismatches and their position on the probe, and must often be determined empirically. For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual (2nd ed.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989) or Current Protocos in Molecular Biology, F.Ausubel et al., ed. Greene Publishing and Wiley-Interscience, New York (1987).

The phrase "nucleic acid sequence encoding" refers to a nucelic acid which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid sequences include both the full length nucleic acid sequences as well as non-full length sequences derived from the full length protein. It being further understood that the sequence includes the degenerate codons of the native sequence or sequences wihich may be introduced to provide codon preference in a specific host cell.

The phrase "isolated" or "substantially pure" refers to nucleic acid preparations that lack at least one protein or nucleic acid normally associated with the nucleic acid in a host cell.

The term "operably linked" as used herein refers to linkage of a promoter upstream from a DNA sequence such that the promoter mediates transcription of the DNA sequence.

The term "vector", refers to viral expression systems, autonomous self-replicating circular DNA (plasmids), and includes both expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is described as hosting an "expression vector", this includes

both extrachromosomal circular DNA and DNA that has been incorporated into the host chomosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

The term "gene" as used herein is intended to refer to a nucleic acid sequence which encodes a polypeptide. This definition includes various sequence polymorphisms, mutations, and/or sequence variants wherein such alterations do not affect the function of the gene product. The term "gene" is intended to include not only coding sequences but also regulatory regions such as promoters, enhancers, and termination regions. The term further includes all introns and other DNA sequences spliced from the mRNA transcript, along with variants resulting from alternative splice sites.

The phrase "recombinant protein" or "recombinantly produced protein" refers to a peptide or protein produced using non-native cells that do not have an endogenous copy of DNA able to express the protein. The cells produce the protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequence. The recombinant protein will not be found in association with proteins and other subcellular components normally associated with the cells producing the protein. The terms "protein" and "polypeptide" are used interchangeably herein.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basisfor a sequence comparison; a reference sequence may be a subset of a larger sequence, for

example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a comparison window may, for example, be conducted by the local homology algorithm of Smith and Waterman Adv.Appl. Math. 2:482 (1981), by the homology alignment algorithm of Needleman and Wunsch J.Mol.Biol. 48:443 (1970), by the search for similarity method of Pearson and Lipman Proc.Natl.Acad. Sci.U.S.A. 85:2444 (1988), or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI).

The terms "substantial identity" or "substantial sequence identity" as applied to nucleic acid sequences and as used herein and denote a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, and more preferably at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence.

As applied to polypeptides, the terms "substantial identity" or "substantial sequence identity" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90

percent sequence identity, more preferably at least 95 percent sequence identity or more. "Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example, "95% amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have 95% amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The phrase "substantially purified" or "isolated" when referring to a peptide or protein, means a chemical composition which is essentially free of other cellular components. It is preferably in a homogeneous state although it can be in either a dry of aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to represent greater than 90% of all macromolecular species present. More preferably the protein is purified to greater than 95%, and most preferably the protein is purified to essential homogeneity, wherein other macromolecular species are not detected by convention techniques.

The phrase "specifically binds to an antibody" or "specifically immunoreactive with", when referring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologies. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein and do not bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactivewith a protein. See Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

Nucleic Acids of the Invention

The instant invention provides PFIC1/BRIC nucleic acids. The nucleic acid sequences of the invention are typically identical to or show substantial sequence identity (determined as described above) to the nucleic acid sequences of Figure 5. Included in this definition are nucleic acids which hybridize to the nucleic acid sequences of Figure 5 under stringent conditions. "Stringent" as used herein refers to hybridization andwash conditions of 50% formamide at 42°C. Other stringent hybridization conditions may also be selected. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and

pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0,02 molar at pH 7 and the temperature is at least about 60°C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one.

Oligonucleotides comprising at least 8-100 consecutive nucleotides unique to the sequence of Figure 5 or its complement, more preferably 15-50 consecutive nucleotides unique to the sequence of Figure 5 or its complement are alspprovided in the instant invention. Such oligonucleotides may be provided as member of primer pairs. Oligonucleotides are useful, for example, for amplification of PFIC1/BRIC sequences, whether genomic or cDNA, and in diagnostic assays.

"Wild-type" or "unaffected" PFIC1/BRIC nucleic acids are identical to or substantially identical to the sequence of Figure 5. These nucleic acids may comprise sequence polymorphisms, silent mutations, or other mutations which do not affect the function of the PFIC1/BRIC protein.

The nucleic acids of the inventions are useful for diagnostic assays to detect, for example, mutations in the PFIC1/BRIC gene. Such mutations may be indicative of aberrant forms of PFIC1/BRIC which result in an affected phenotype in a patient. Exemplary mutations include but are not limited to G308V, G892R, L288S, 2097-2(T-C), I1661T, 795delGNR, and 2286-4, -3(CT-AA). Various formats for such assays are described in detail below.

The nucleic acids are typically deliverd to cells

by the methods described in more detail below. In some embodiments, expression of the polypeptides encoded by the nucleic acids is expected to prevent, ameliorate, or lessen the symptoms of cholestasis. Whether provided via nucleic acid or polypeptides delivered directly to cells, the therapeutic formulations of the invention can also be use as adjuncts to other forms of therapy.

Such nucleic acids may encode the wild-type, or unaffected gene product, or may encode antagonists or agonists of PFIC1/BRIC gene in the host cell. An antagonist of PFIC1/BRIC is an agent which enhances symptoms of cholestasis. An agonist of PFIC1/BRIC is an agent which prevents, ameliorates, or lessons the symptoms of cholestasis. A therapeutic dose of an agonist of the invention is a dose sufficient to prevent, ameliorate, or lesson the symptoms of cholestasis.

Typically, patients with benign recurrent intrahepatic cholestasis (BRIC) and progressive familial intrahepatic cholestasis type 1 (PFIC1)are preferred candidates for treatmentwith the compositions of the invention, although patients with other liver diseases involving cholestasis may also be so treated.

Polypeptides of the Invention

The polypeptides of the invention include the polypeptides encoded by the nucleic acid of Figure 5, amino acid substitutionsthereof which do not affect function of the PFIC1/BRIC protein, biologically active fragments thereof, and mutants orvariants thereof which can serve as agonists or antagonists of PFIC1/BRIC.

The polypeptides of the invention are useful, for example, in the therapy of cholestasis as discussed above and in screening for and isolation of co-activators, accessory proteins, or targets of the PFIC1/BRIC protein.

In some embodiments, administration of a polypeptide, for example, is expected to prevent, ameliorate, or lessen the symptoms of cholestasis. Such polypeptides may comprise the full length wild-type, or unaffected gene product, fragments thereof, or agonists of PFIC1/BRIC in the host cell. Antagonists and agonists are discussed above, as are exemplary indications for treatment with the polypeptides of the invention.

The polypeptides of the invention are also useful for the generation of and screening of antibodies. Such antibodies are useful, for example, as antagonists of PFIC1/BRIC protein, in the purification of the polypeptides of the invention, and in diagnostic assays for PFIC1/BRIC protein.

Agonists and Antagonists of PFIC1/BRIC

As discussed above, in some embodiments of the invention, antagonists and agonists of PFIC1/BRIC are provided. Such antagonists or agonists include but are not limited to antibodies which specifically bind tot PFIC1/BRIC; antibodies which specifically bind to an PFIC1/BRIC do-activator, accessory protein, or target; mutants of PFIC1/BRIC protein; antisense PFIC1/BRIC nucleic acids; co-activators or accessory proteins for PFIC1/BRIC; and peptide, non-peptide, and peptidomimetic analogs of such co-activators, accessory proteins, or targets or of the PFIC1/BRIC polypeptide.

Agonists of PFIC1/BRIC include the wild-type PFIC1/BRIC polypeptide and may also comprise mutants of PFIC1/BRIC.

Ligands of PFIC1/BRIC

As used herein, "ligand" means a molecule that is recognized by a particular protein. The agent bound by of reacting with the protein is called a "ligand", a term which

is definitionally meaningful only in terms of its counterpart protein. The term "ligand" does not imply any particular molecular size or other structural or compositional feature other than that the substance in question is capable of binding or otherwise interacting with the protein. Also, a "ligand" may serve either as the natural ligand to which the PFIC1/BRIC polypeptide binds or interacts, or as a functional analogue that may act as an agonist or antagonist. Thus, the present invention provides methods for the affinity purification of ligands that interact with the PFIC1/BRIC. Ligands that can be investigated by this invention include but are not restricted to, agonists and antagonists, toxins and venoms, viral epitopes, hormones, sugars, cofractors, peptides, enzyme substrates, cofactors, drugs, and proteins.

Furthermore, peptide and protein ligands can be obtained by "panning" peptide or protein libraries displayed on filamentous bacteriophage against immobilized PFIC1/BRIC protein (for examples of techniques, see Roberts et al., Gene 121:9-15 (1992); Dennis et al., J.Biol.Chem 270:25411-25417 (1995); Wang et al., J.Biol.Chem. 270:12250-12256 (1995)). Briefly, bound phage are eluted at low pH and amplified in host cells from which the PFIC1/BRIC-binding peptide sequences can be recoverd by PCR. Ligands may also be identified using the yeast two-hybrid system (Fields et al., Nature 340:245-247 (1989)). The PFIC1/BRIC-binding peptides can then be tested for their ability to inhibit a PFIC1/BRIC activity.

General Methods

The nucleic acid compositions of this invention, whether RNA, cDNA, genomic DNA, or a hybrid of the various combinations, may be isolated from natural sources, including cloned DNA, or may be synthesized in vitro. The nucleic acids claimed may be present in transformed or transfected

whole cells, in a transformed or transfected cell lysate, or in a partially purified or substnatially pure form.

Tebniques for nucleic acid manipulation of the nucleic acid sequences of the invention such as subcloning nucleic acid sequences encoding polypeptides into expression vectors, labeling probes, DNA hybridization, and the like are described generally in Sambrook et al., Molecular Cloning - A Laboratory Manual (2nd Ed.), vol.1-3, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1989), which is incorporated herein by reference. This manual is hereinafter referred to as "Sambrook et al.".

There are various methods of isolating the nucleic acid sequences of the invention. For example, DNA is isolated from a genomic or cDNA library using labeld oligonucleotide probes having sequences complementary to the sequences disclosed herein. Such probes can be used directly in hybridization assays. Alternatively probes can be designed for use in amplification techniques such as PCR.

To prepare a cDNA library, mRNA is isolatedfrom tissue such as heart of pancreas, preferably a tissue wherein expression of the gene or gene family is likely to occur. cDNA is preparedfrom the mRNA and ligated into a recombinant vector. The vector is transfected into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known. See Gubler, U. and Hoffman, B.J. Gene 25:263-269 (1983) and Sambrook et al.

For a genomic library, the DNA is extracted from tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 KB. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged in vitro, as described in Sambrook, et al. Recombinant phage are analyzed by plaque

hybridization as described in Benton and Davis, Science 196:180-182, (1977). Colony hybridization is carried out as generally described in M.Grunstein et al., Proc.Natl.Acad. Sci.USA 72:3961-3965 (1975).

DNA of interest is identified in either cDNA or genomic libraries by its ability to hybridize with nucleic acid probes, for example on Southern blots, and these DNA regions are isolated by standard methods familiar to those of skill in the art. See Sambrook et al.

Various methods of amplifying target sequences, such as the polymerase chain reaction, can also be used to prepare DNA. Polymerase chain reaction (PCR) technology is used to amplify such nucleic acid sequences directly from mRNA, from cDNA, and from genomic libraries or cDNA libraries. The isolated may also be used as templates for PCR amplification.

In PCR techniques, oligonucleotide primers complementary to the two 3' borders of the DNA region to be amplified are synthesized. The polymerase chain reaction is then carried out using the two primers. See PCR Protocols: A Guide to Methods and Applications ((Innis, M, Gelfland,D., Sninsky, J. and White, T., ed.), Academic Press, San Diego (1990)). Primers can be selected to amplify the entire regions encoding a full-length sequence of interest or to amplify smaller DNA segments as desired.

PCR can be used in a variety of protocols to isolate cDNA's encoding a sequence of interest. In these protocols, appropriate primers and probes for amplifying DNA encoding a sequence of interest are generated from analysis of the DNA sequences listed herein. Once such regions are PCR-amplified, they can be sequenced and oligonucleotide probes can be prepared from the sequence obtained.

Oligonucleotides for use as probes are chemically synthesized according to the solid phase phosphoramidite

triester method first described by Beaucage et al., Tetrahedron Lett., 22(20):1859-1862 (1981) using an automated synthesizer, as described in Needham-VanDevanter et al., Nucleic Acids Res. 12:6159-6168 (1984). Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange HPLC as described in Pearson, J.D. and Regnier, F.E., J.Chrom., 255:137-149 (1983). The sequence of the synthetic oligonucleotide can be verified using the chemical degradation method of Maxam, A.M. and Gilbert, W., in Grossman, L. and Moldave, D., eds., Academic Press, New York, Methods in Enzymology 65:499-560 (1980).

A. **Expression**

Once DNA encoding a sequence of interest is isolated and cloned, one can express the encoded proteins in a variety of recombinantly engineered cells. It is expected that those of skill in the art are knwledgeable in the numerous expression systems available for expression of DNA encoding A. No attempt to describe in detail the various methods known for the expression of proteins in prokaryotes or eukaryotes is made here.

In brief summary, the expression of natural of synthetic nucleic acids encoding a sequence of interest will typically be achieved by operably linking the DNA of cDNA to a promoter (which is either constitutive or inducible) followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of polynucleotide sequence of interest. To obtain high level expression of a cloned gene, it is desirabel to construct expression plasmids which contain, at the minimum,

a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/ translation terminator. The expression vectors may also comprise generic expression cassettes containg at least one independent terminator sequence, sequences permitting replication of the plasmid in both eukaryotes and prokaryotes i.e., shuttle vectors, and selection markers for both prokaryotic and eukaryotic systems.

1. **Expression in Prokaryotes**

A variety of procaryotic expression systems may be used to express the proteins of the invention. Examples include E.coli, Bacillus, Streptomyces, and the like.

It is preferred to construct expression plasmids which contain, at the minimum, a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. Examples of regulatory regions suitable for this purpose in E.coli are the promoter and operator region of the E.coli tryptophan biosynthetic pathways as described by Yanofsky, J.Bacteriol. 158:1018-1024 (1984) and the leftward promoter of phage lambda (Pλ) as described by Herskowitz, I and Hagen, D., Ann.Rev.Genet. 14:399-445 (1980). The inclusion of selection markers in DNA vectors transformed in E.coli is also useful. Examples of such markers include genes specifying resistance to ampicillin, tetracycline, or chloramphenicaol. See Sambrook et al. for details concerning selection markers for use in E.coli.

To enhance proper folding of the expressed recombinant protein, during purification from E.coli, the expressed protein may first be denatured and then renatured. This can be accomplished by solubilizing the bacterially produced proteins in a chaotropic agent such as guanidine HCl and reducing all the cysteine residues with a reducing

agent such as beta-mercaptoethanol. The protein is then renatured, either by slow dialysis or by gel filtration. See U.S.Patent No. 4,511,503.

Detection of the expressed antigen is achieved by methods known in the art as radioimmunoassay, or Western blotting techniques or immunoprecipitation. Purification from E.coli can be achieved following procedures such as those described in U.S.Patent No. 4,511,503.

2. **Expression in Eukaryotes**

A variety of eukaryotic expression systems such as yeast, insect cell lines, bird, fish, and ammalian cells, are known to those of skill in the art. As explained briefly below, a sequence of interest may be expressed in these eukaryotic systems.

Synthesis of heterologous proteins in yeast is well known. Methods in Yeast Genetics, Sherman, F., et al., Cold Spring Harbor Laboratory, (1982) is a well recognized work describing the various methods available to produce the protein in yeast.

Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired. For instance, suitable vectors are described in the literature (Botstein, et al., Gene 8:17-24 (1979); Broach, et al., Gene 8:121-133 (1979)).

Two procedures are used in transforming yeast cells. In one case, yeast cells are first converted into protoplasts using zymolyase, lyticase or glusulase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are given in the papers by J.D.Beggs, Nature(London) 275:104-109

(1978); and Hinnen, A., et al., Proc.Natl.Acad.Sci.U.S.A. 75:1929-1933 (1978). The second procedure does not involve removal of the cell wall. Instead the cells are treated with lithium chloride or acetate and PEG and put on selective plates (Ito, H., et al., J.Bact. 153:163-168 (1983)).

The proteins of the invention, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay of other standard immunoassay techniques.

The sequences encoding the proteins of the invention can also be ligated to various expression vectors for use in transforming cell cultures of, for instance, mammalian, insect, bird or fish origin. Illustrative of cell cultures useful for the production of the polypeptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells although mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines, and various human cells such as COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can include expression control sequences, such as an origin ofreplication, a promoter (e.g., the CMV promoter, a HSV tk promoter or pgk (phosphoglycerate kinase) promoter), an enhancer (Queen et al. Immunol.Rev. 89:49 (1986)), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T Ag poly A addition site), and transcriptional terminator sequences. Other animal cells useful for production ofproteins are available, for instance, from the American Type Culture Collection Catalogue of

Cell Lines and Hybridomas (7th edition, (1992)).

Appropriate vectors for expressing the proteins of the invention in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth and Drosophila cell lines such as a Schneider cell line (see Schneider J.Embryol.Exp.Morphol 27:353-365 (1987).

As indicated above, the vector, e.g., a plasmid, which is used to transform the host cell, preferably contains DNA sequences to initiate transcription and sequences to control the translation of the protein. These seuqneces are referred to as expression control sequences.

As with yeast, when higher animal host cells are emplyed, polyadenylation or transcription terminator sequenced from known mammalian genes need to be incorporated into te vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is theVP1 intron from SV40 (Sprague, J. et al., J.Virol. 45:773-781 (1983)).

Additionally, gene sequences to control replication in the host cell may be incorporated into the vector such as those found in bovine papilloma virus type-vectors. Saveria-Campo, M., 1985, "Bovine Papilloma virusDNA a Eukaryotic Cloning Vector" in DNA Cloning Vol. II a Practical Approach Ed.D.M.Glover, IRL Press, Arlington, Virginia pp. 213-238.

The host cells are competent orrendered competent for transformation by various means. There are several well-known methods of introductin DNA into animal cells. These include: calcium phosphate precipitation, fusion of the recipient cells with bacterial protoplasts containing

the DNA, treatment of the recipient cells with liposomes containing the DNA, DEAE dextran, electroporation and micro-injection of the DNA directly into the cells.

The transformed cells are cultures by means well known in the art. Biochemical Methods in Cell Culture and Virology, Kuchler, R.J., Dowden, Hutchinson and Ross, Inc., (1977). The expressed polypeptides are isolated from cells grown as suspensions or as monolayers. The latter are recovered by well known mechanical, chemical of enzymatic means.

B. **Purification**

The proteins produced by recombinant DNA technology may be purified by standard techniques well known to those of skill in the art. Recombinantly produced proteins can be directly expressed or expressed as fusion proteins. The protein is then purified by a combination of cell lysis (e.g., sonication) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired polypeptide.

The polypeptides of this invention may be purified to substantial purity by standard techniques well known in the art, including selective precipitation with such substances as ammonium sulfate, column chromatography, immunopurification methods, and others. See, for instance, R.Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982), incorporated herein by reference. For example, antibodies may be raised to the proteins of the invention as described herein. Cell membranes are isolated from a cell line expressing the recombinant protein, the protein is extracted from the membranes and immunoprecipitated. The proteins may then be further purified by standard protein chemistry techniques as

described above.

C. **In Vitro Diagnostic Methods**

The present invention provides methods for detecting DNA or RNA encoding the proteins of the invention and for measuring the proteins by immunoassay techniques. These methods are useful for two general purposes. First, assays for detection of nucleic acids encoding the proteins of the invention are useful for the isolation of these nucleic acids from a variety of vertebrate species according to the methods dexcribed in section (B) above and by use of the nucleic acid hybridization assays described below.

The nucleic acid hybridization assays and the immunoassays described below are also useful as in vitro the invention in a sample involves a Southern transfer. Briefly, the digested genomic DNA is run on agarose slab gels in buffer and transferred to membranes. Hybridization is carried out using the nucleic acid probes discussed above. As described above, nucleic acid probes are designed based on the nucleic acid sequences of the invention. The probes can be full length or less than the full length of the nucleic acid sequence encoding FIC1. Shorter probes are empirically tested for specificity. Preferably nucleic acid probes are 20 bases or longer in length. Visualization of the hybridized portions allows the qualitative determination of the presence or absence of DNA encoding the cholestation protein.

Similarly, a Northern transfer may be used for the detection of mRNA encoding the proteins of the invention. In brief, the mRNA is isolated from a given cell sample using an acid guanidinium-phenol-chloroform extraction method. The mRNA is then electrophoresed to separate the mRNA species and the mRNA is transferred from the gel to a nitrocellulose membrane. As with the Southern blots, labeled

probes are used to identify the presence or absence of the proteins of the invention.

A variety of nucleic acid hybridization formats are known to those skilled in the art. For example, common formats include sandwich assays and competition or displacement assays. Hybridization techniques are generally described in Nucleic Acid Hybridization: A Practical Approach Ed. Hames, B.D. and Higgins, S.J., IRL Press (1985); Gall et al., Proc.Natl.Acad.Sci.U.S.A. 63:378-383 (1969); and John et al., Jones Nature 223:582-587 (1969).

For example, sandwich assays are commercially useful hybridization assays for detecting or isolating nucleic acid sequences. Such assays utilize a "capture" nucleic acid covalently immobilized to a solid support and a labeled "signal" nucleic acid in solution. The clinical sample will provide the target nucleic acid. The "capture" nucleic acid and "signal" nucleic acid robe hybridize with the target nucleic acid to form a "sandwich" hybridization complex. To be effective, the signal nucleic acid cannot hybridize with the capture nucleic acid.

Typically, labeled signal nucleic acids are used to detect hybridization. Complementary nucleic acids or signal nucleic acids may be labeled by any one of several methods typically used to detect the presence of hybridized polynucleotides. The most common method of detection is the use of autoradiography with ^{3}H, ^{125}I, ^{35}S, ^{14}C, of ^{32}P-labeled probes or the like. Other labels include ligands which bind to labeled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labeled ligand.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such

binding occurs through ligand and anti-ligang interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal.

The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or, in some cases, by attachment to a radioactive label. (Tijssen, P., "Practice and Theory of Enzyme Immunoassays", Laboratory Techniques in Biochemistry and Molecular Biology Burdon, R.H., van Knippenberg, P.H., Eds., Elsevier (1985), pp.9-20.)

The sensitiviy of the hybridization assays may be enhanced through use of a nucleic acid amplification system which multiplies the target nucleic acid being detected. Examples of such systems include the polymerase chain reaction (PCR) system and the ligare chain reaction (LCR) system. Other methods recently described in the art are the nucleic acid sequence based amplification (NASBA™, Cangene, Mississauga, Ontario) and Q Beta Replicase systems.

An alternative means for determining the level of expression of a gene encoding the proteins of the invention is in situ hybridization. In situ hybridization assays are well known and are generally described in Angerer et al., Methods Enzymol. 152:649-660 (1987). In an in situ hybridization assay, cells are fixed to a solid support, typically a glass slide. If DNA is to be probed, the cells are denatures with heat or alkali. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of labeled probes specific to the proteins of the invention. The probes are preferably labeled with radioisotopes or fluorescent reporters.

Methods of screening nucleic acid for mutations are well known in the art, including, but not limited to, restriction-fragment-length-polymorphism detection based on allele-specific restriction-endonuclease cleavage (Kan et al., Lancet ii:910-912 (1978)), hybridization with allele-specific oligonucleotide probes (Wallace et al., Nucl.Acids Res. 6:3543-3557 (1978)), including immobilized oligonucleotides (Saiki et al., Proc.Natl.Acad.Sci.U.S.A. 86:6230-6234 (1989)) or oligonucleotide arrays (Maskos et al. Nucl.Acids Res. 21:2260-2270 (1993)), allele-specific PCR (Newton et al., Nucl.Acids Res. 17:2503-2516 (1989)), mismatch-repair detection (MRD) (Faham, Genome Res. 5:474-482 (1995)), binding of MutS protein (Wagner et al., Nucl. Acids Res. 23:3944-3948 (1995)), denaturing-gradient gel electrophoresis (DGGE) (Fisher et al., Proc.Natl.Acad.Sci. U.S.A. 80:1579-1583 (1983)), single-strand-conformation-polymorphism detection (Orita et al., Genomics 5:874-879 (1983)), RNAase cleavage at mismatched base-pairs (Myers et al., Science 230:1242 (1985)), chemical (Cotton et al., Proc.Natl.Acad.Sci.U.S.A. 85:4397-4401 (1988)) or enzymatic (Youil et al., Proc.Natl.Acad.Sci.U.S.A. 92: 87-91 (1995)), cleavage of heteroduplex DNA, methods based on allele specific primer extension (Syvänen et al., Genomics 8:684-692 (1990)), genetic bit analysis (GBA) (Nikiforov et al., Nucl.Acids Res. 22:4167-4175 (1994)), the oligonucleotide-ligation assay (OLA) (Landegren et al., Science 241:1077 (1988)), the allele-specific ligation chain reaction (LCR) (Barany, Proc.Natl.Acad.Sci.U.S.A. 88:189-193 (1991)), gap-LCR (Abravaya et al., Nucl.Acids.Res. 23:675-682 (1995)), and radioactive and/or fluorescent DNA sequencing using standard procedures well known in the art.

D. **Production of Antibodies and Development of Immunoassays**

Immunoassays can be used to qualitatively or quantitatively analyze for the proteins of the invention. A general overview of the applicable technology can be found in Harlow and Lane, Antibodies: A Laboratory Manual, Cold Spring Harbor Pubs., N.Y. (1988), incorporated herein by reference.

1. **Antibody Production**

A number of immunogens may be used to produce antibodies specifically reactive with the proteins of the invention. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Naturally occurring protein may also be used either in pure or impure form. Synthetic peptides made using the protein sequences described herein may also used as an immunogen for the production of antibodies to the protein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described aove. The product is then injected into an animal capable of producing antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. In brief, an immunogen, preferably a purified protein, is mixed with an adjuvant and animals are immunized. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired. (See Harlow and Lane, supra).

Monoclonal antibodies may be obtained by various

techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see Kohler et al., Eur.J.Immunol. 6:511-519 (1976), incorporated herein by reference). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Juse, et al., Science 246:1275-1281 (1989).

Methods of production of synthetic peptides are known to those of skill in the art. Peptides preferably at least 10 amino acids in length are synthesized corresponding to these regions and the peptides are conjugated to larger protein molecules for subsequent immunization. Preferably, peptide sequences corresponding to regions of interest of a recombinant protein of the invention is used to generate antibodies specifically immunoreactive with the protein. Production of monoclonal or polyclonal antibodies is then carried out as described above.

2. **Immunoassays**

A particular protein can be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures in general, see Basic and Clinical Immunology 7th Edition (D.Stites and A.Terr ed.) (1991). Moreover, immunoassays can be performed in any of several

configurations, which are reviewed extensively in Enzyme Immunoassay, E.T.Maggio, ed., CRC Press, Boca Raton, Florida (1980); "Practice and Theory of Enzyme Immunoassays", P.Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers B.V. Amsterdam (1985); and Harlow and lane, Antibodies, A Laboratory Manual, supra, each of which is incorporated herein by reference.

Immunoassays for measurement of the proteins of the invention can be performed by a variety of methods know to those skilled in the art. In brief, immunoassays to measure the protein can be either competitive or noncompetitive binding assays. In competitive binding assays, the sample analyte competes with a labeled analyte for specific binding sites on a capture agent bound to a solid surface. Preferably the capture agent is an antibody specifically reactive with a recombinant protein of the invention produced as described above. The concentration of labeled analyte bound to the capture agent is inversely proportional to the amount of free analyte present in the sample.

Western blot analysis can also be done to determine the presence of a protein of the invention in a sample. Electrophoresis is carried out, for example, on a tissue sample suspected of containing the protein. Following electrophoresis to separate the proteins, and transfer of the proteins to a suitable solid support such as a nitro-cellulose filter, the solid support is then incubated with an antibody reactive with the protein. This antibody may be labeled, or alternatively may be it may be detected by subsequent incubation with a second labeld antibody that binds the primary antibody.

The immunoassay formats described above employ labeled assay components. The label can be in a variety of

forms. The label may be coupled directly of indirectly to the desired component of the assay according to methods well known in the art. A wide variety of labels may be used. The component may be labeled by any one of several methods. Traditionally a radioactive label incorporation ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P was used. Non-radioactive labels include ligands which bind to labeled antibodies, fluorophores, chemiluminescent agents, enzymes, and antibodies which can serve as specific binding pair members for a labeled ligand. The choice of label depends on sensitivity required, ease of conjugation with the compound, stability requirements, and available instrumentation. For a review of various labeling or signal producing systems which may be used, see U.S.Patent No.4,391,904, which is incorporated herein by reference.

Antibodies reactive with a particular protein can also be measured by a variety of immunoassay methods. For a review of immunological and immunoassay procedures applicable to the measurement of antibodies by immunoassay techniques, see Basic and Clinical Immunology 7th Edition (D.Stites and A.Terr ed.) supra, Enzyme Immunoassay, E.T.Maggio, ed., supra, and Harlow and Lane, Antibodies, A Laboratory Manual, supra.

Kits

This invention also embraces kits for detecting the presence of a protein of the invention in tissue or blood samples which comprise a container containing antibodies selectively immunoreactive to the protein and instructional material for performing the test. The kit may also contain other components such as a protein of the invention, controls, buffer solutions, and secondary antibodies. Kits for detecting antibodies to a protein of the invention comprise a container containing an a protein of the invention,

instructional material and may comprise other materials such as secondary antibodies and labels as described herein.

This invention further embraces diagnostic kits for detecting DNA or RNA encoding proteins of interest in tissue or blood samples which comprise nucleic probes as described herein and instructional material. The kit may also contain additional component such as labeled compounds, as described herein, for identification of duplexed nucleic acids.

In some embodiments of the invention, a kit for treatment of cholestasis is provided. Such kits will contain polynucleotides for gene therapy or polypeptides for administration to a patient in need of treatment. Such kits may contain such items as buffers and other reagents for formulating the polynucleotides or polypeptides and directions for use.

Formulations

The compositions of the invention will be formulated for administration by manners known in the art acceptable for administration to a mammalian subject, preferably a human. In some embodiments of the invention, the compositions of the invention can be administered directly into a tissue by injection or into a blood vessel supplying the tissue of interest. In further embodiments of the invention the compositions of the invention are administered "locoregionally", i.e., intravesically, intralesionally, and/or topically. In other embodiments of the invention, the compositions of the invention are administered systemically by injection, inhalation, suppository, trans-dermal delivery, etc. In further embodiments of the invention the compositions are administered through catheters or other devices to allow access to a remote tissue of interest, such as an internal organ. The compositions of the invention can also be administered in depot type devices,

implants, or encapsulated formulations to allow slow or sustained release of the compositions.

In order to administer therapeutic agents based on, or derived from, the present invention, it will be appreciated that suitable carriers, excipients, and other agents may be incorporated into the formulations to provide improved transfer, delivery, tolerance, and the like.

A multitude of appropriate formulations can be found in the formulary known to all pharmaceutical chemists: Remington's Pharmaceutical Sciences, (15th Edition, Mack Publishing Company, Easton, Pennsylvania (1975)), particularly Chapter 87, by Blaug, Seymour, therein. These formulations include for example, powders, pastes, ointments, jelly, waxes, oils,lipids, anhydrouw absorption bases, oil-in-water or water-in-oil emulsions, emulsions carbowax (polyethylene glycols of a variety of molecular weights), semi-solid gels, and semi-solid mixtures containing carbowax.

Any of the foregoing formulations may be appropriate in treatments and therapies in accordance with the present invention, provided that the active agent in the formulation is not inactivated by the formulation and the formulation is physiologically compatible.

The quantities of active ingredient necessary for effective therapy will depend on many different factors, including means of administration, target site, physiological state of the patient, and other medicaments administered. Thus, treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of the active ingredients. Animal testing of effective doses for treatment of particular disorders will provide further predictive indication of human dosage. Various considerations are described, for example, in

Goodman and Gilman's the Pharmacological Basis of Therapeutics, 7th Edition (1985), MacMillan Publishing Company, New York, and Remington's Pharmaceutical Sciences 18th Edition, (1990) Mack Publishing Co., Easton Penn. Methods for administration are discussed therein, including oral, intravenous, intraperitoneal, intramuscular, transdermal nasal, iontophoretic administration, and the like.

The compositions of the invention may be administered in a variety of unit dosage forms depending on the method of administration. For example, unit dosage forms suitable for oral administration include solid dosage forms such as powder, tablets, pills, capsules, and dragees, and liquid dosage forms, such as elixirs, syrups, and suspensions. The active ingredients may also be administered parenterally in sterile liquid dosage forms. Gelatin capsules contain the active ingredient and as inactive ingredients powdered carriers, such as glucose, lactose, sucrose, mannitol, starch, cellulose or cellulose derivatives, magnesium stearate, stearic acid, sodium saccharin, talcum, magnesium carbonate and the like. Examples of additional inactive ingredients that may be added to provide desirable color, taste, stability, buffering capacity, dispersion or other known desirable features are red iron oxide, silica gel, sodium lauryl sulfate, titanium dioxide, edible white ink and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactures as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric-coated for selective disintegration in the gastrointestinal tract. Liquid dosage forms for oral administration can contain coloring and flavoring to

increase patient acceptance.

The concentration of the compositions of the invention in the pharmaceutical formulations can vary widely, i.e., from less than about 0.1%, usually at or at least about 2% to as much as 20% to 50% or more by weight, and will be selected primarily bu fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected.

The compositions of the invention may also be administered via liposomes. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. In these preparations the composition of the invention to be delivered is incorporated as part of a liposome, alone or in conjunction with a molecule which binds to a desired target, such as antibody, or with other therapeutic or immunogenic compositions. Thus, liposomes either filled or decorated with a desired composition of the invention can delivered systemically, or can be directed to a tissue of interest, where the liposomes then deliver the selected therapeutic/immunogenic peptide compositions.

Liposomes for use in the invention are formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of, e.g., liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka et al. Ann.Rev.Biophys.Bioeng. 9:467 (1980), U.S.Patent Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369, incorporated herein by reference.

A liposome suspension containing a composition of the invention may be administered intravenously, locally,

topically, etc. in a dose which varies according to, inter alia, the manner of administration, the composition of the invention being delivered, and the stage of the disease being treated.

For solid compositions, conventional nontoxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceutically acceptale nontoxic composition is formed by incorporating any of the normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, one of more compositions of the invention of the invention, and more preferably at a concentration of 25%-75%.

For aerosol administration, the compositions of the invention are preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of compositoins of the invention are 0.01%-20% by weight, preferably 1%-10%. The surfactant must, of course, be nontoxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1%-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. A carrier can also be included, as desired, as with, e.g., lecithin for intranasal delivery.

The compositions of the invention can additionally

be delivered in a depot-type system, an encapsulated form, or an implant by techniques well-known in the art. Similarly, the compositions can be deliverd via a pump to a tissue of interest.

The compositions of the invention are typically administered to pateints after the onset of symptoms, although treatment can also be prophylactic in some embodiments. Typically, treatment with direct administration of polypeptides is done daily, weekly, or monthly, for a period of time sufficient to reduce, prevent, or ameliorate symptoms. Treatment with the nucleic acids of the invention is tyically done at intervals of several months. In some embodiments, administration of the compositions of the invention is done in utero.

A therapeutic dose or amount of the compositions of the invention is a dose or amount sufficient to prevent, lessen, or ameliorate symptoms of cholestasis.

Gene Therapy

Gene therapy utilizing recombinant DNA technology to deliver nucleic acids encoding PFIC1/BRIC polypeptides, or antagonists or agonists of PFIC1/BRIC into patient cells or vectors which will supply the patient with gene product in vivo is also contemplated within the scope of the present invention.

Gene therapy techniques have the potential for limiting the exposure of a subject to a gene product, such as a PFIC1/BRIC polypeptide, by targeting the expression of the therapeutic gene to a tissue of interest, such as skeletal muscle, myocardium, vascular endothelium or smooth muscle, or solid or circulating tumor cells. For example, WIPO Patent Application Publication No. WO 93/15609 discloses the delivery of interferon genes to vascular tissue by administration of such genes to areas of vessel

wall injury using a catheter system. In another example, an adenoviral vector encoding a protein capable of enzymatically converting a prodrug, a "suicide gene", and a gene encoding a cytokine are administered directly into a solid tumor.

Other methods of targeting therapeutic genes to tissues of interest include the three general categories of transductional targeting, positional targeting, and transcriptional targeting (for a review,see, e.g., Miller et al. FASEB J. 9:190-199 (1995)). Transductional targeting refers to the selective entry into specific cells, achieved primarily by selection of a receptor ligang. Positional targeting within the genome refers to integration into desirable loci, such as active regions of chromatin, or through homologous recombination with an endogenous nucleotide sequence such as a target gene. Transcriptional targeting refers to selective expression attained by the incorporation of transcriptional promoters with highly specific regulation of gene expression tailored to the cells of interest.

Examples of tissue-specific promoters include a liver-specific promoter (Zou et al., Endocrinology 138: 1771-1774 (1997)); a small intestine-specific promoter (Olivera et al., J.Biol.Chem. 271:31831-31838 (1996)); the promoter for creatine kinase, which has been used to diret the expression of dystrophin cDNA expression in muscle and cardiac tissue (Cox et al., Nature 364:725-729 (L993)); and immunoglobulin heavy or light chain promoters for the expression of suicide genes in B cells (Maxwell et al., Cancer res. 51:4299-4304 (1991)). An endothelial cell-specific regulatory region has also been characterized (Jahroudi et al., Mol.Cell.Biol. 14:999-1008 (1994)). Amphotrophic retroviral vectors have been constructed carrying a herpes simplex virus thymidine kinase gene under

the control of either the albumin or alpha-feotprotein promoters (Huber et al., Proc.Natl.Acad.Sci., U.S.A. 88:8039-8043 (1991)) to target cells of liver lineage and hepatoma cells, respectively. Such tissue specific promoters can be used in retroviral vectors (Hartzoglou et al., J.Biol.Chem. 265:17285-17293 (1990)) and adenovirus vectors (Friedman et al., Mol.Cell.Biol. 6:3791-3797 (1986)) and stil retain their tissue specificity.

Other elements aiding specificity of expression in a tissue of interest can include secretoin leader sequences, enhancers, nuclear localization signals, endosmolytic peptides, etc. Preferably, these elements are derived from the tissue of interest to aid specificity.

Viral vector systems useful in the practice of the instant invention include but are not limited to adenovirus, herpesvirus, adeno-associated virus, minute virus of mice (MVM), HIV, sindbis virus, and retroviruses such as Rous sarcoma virus, and MoMLV. Typically, the nucleic acid encoding the therapeutic polypeptide of interest is inserted into such vectors to allow packaging of the nucleic acid, typically with accompanying viral DNA, infection of a sensitive host cell, and expression of the polypeptide of interest.

In still other embodiments of the invention, nucleic acid encoding a therapeutic polypeptide of interest is conjugated to a cell receptor ligand for faciliatated uptake (e.g., invagination of coated pits and internalization of the endosome) through a DNA linking moiety (Wu et al., J.Biol.Chem. 263:14621-14624 (1988); WO 92/06180). For example, the DNA constructs of the invention can be linked through a polylysine moiety to asialo-oromucoid, which is a ligand for the asialoglycoprotein receptor of hepatocytes.

Similarly, viral envelopes used for packaging the

recombinant constructs of the invention can be modified by the addition of receptor ligands or antibodies specific for a receptor to permit receptor-mediated endocytosis into specific cells (e.g., WO 93/20221, WO 93/14188; WO 94/06923). In some embodiments of the invention, the DNA constructs of the invention are linked to viral proteins, such as adenovirus particles, to facilitate endocytosis (Curiel et al., Proc.Natl.Acad.Sci.U.S.A. 88:8850-8854 (1991)). In other embodiments, molecular conjugates of the instant invention can includemicrotubule inhibitors (WO 94/06922); synthetic peptides mimicking influenza virus hemagglutinin (Plank et al., J.Biol.Chem. 269:12918-12924 (1994)); and nuclear localization signals such as SV40 T antigen (WO 93/19768).

The nucleic acid can be introduced into the tissue of interest in vivo or ex vivo by a variety of methods. In some embodiments of the invention, the nucleic acid is introduced to cells by such methods as microinjection, calcium phosphate precipitation, liposome fusion, or biolistics. In further embodiments, the nucleic acid is tken up directly by the tissue of interest. In other embodiments, nucleic acid is packages into a viral vector system to facilitate introduction into cells.

In some embodiments of the invention, the compositions of the invention are administered ex vivo to cells or tissues explanted from a patient, then returned to the patient. Examples of ex vivo administration of gene therapy constructs include Arteaga et al., Cancer Research 56(5):1098-1103 (1996); Nolta et al., Proc.Natl.Acad.Sci. USA 93(6):2414-9 (1996); Koc et al., Seminars in Oncology 23(1):46-65 (1996); Raper et al., Annals of Surgery 223(2): 116-26 (1996); Dalesandro et al., J.Thorac.Cardi.Surg. 11(2):416-22 (1996); and Makarov et al., Proc.Natl.Acad.Sci.

USA 93(1):402-6 (1996).

The following examples are provided to illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims.

Experimental Examples

The gene mutated in BRIC was mapped to 18q21-q22, using a search for shared genome segments in three distantly related affected Dutch individuals (Houwen et al., Nat.Genet. 8:380 (1994)). Linkage analysis in a larger set of families confirmed this localization (Sinke, et al., Hum.Genet. 100:382 (1997)). The PFIC1 locus was mapped to an overlapping interval through identification of a haplotype for which two distantly related Amish PFIC1 patients were homozygous; this result suggested that both diseases are caused by mutations in the same gene (Carlton et al., Hum.Mol.Genet. 4:1049 (1995)).

We refined the localization of both BRIC and PFIC1 to the same interval of less than one centiMorgan (cM) (D18S41-D18S849) through examination of haplotype sharing in expanded sets of patients with each disorder. A microdeletion spanning the region containg markers D18S381, D18S849, and D18S1144 on a single chromosome in PFIC1 patient S.K. further narrowed the candidate region to an interval of less than 300 KB, between D18S977/D18S401 and D18S849, as shown in Fig.1A. For that study, patients were designated as having PFIC1 if the displayedextended marker haplotypes in the PFIC1/BRIC candidate region that were either homozygous or that were conserved with the haplotypes of multiple other patients.

We assembled a contig spanning the candidate region, consisting of yeast artificial chromosome (UAC), P1, P1 artificial chromosome (PAC), and bacterial artificial

chromosome (BAC) clones (Figure 1B). To identify genes in the candidate region, we used three approaches: examination of previously mapped expressed sequence tags (ESTs), sequence scanning, and hybridization of a PAC insert (from PAC 52L12, see Figure 1B) to a liver cDNA library. We initiated these gene-identification approaches after an extensive search revealed no evidence in the PFIC1/BRIC candidate region of any ATP-binding cassette (ABC) protein genes. The hypothesis that the PFIC1/BRIC gene would be a member of this gene family was based on the assumption that these disorders were likely due to defective transport of bile acids across the canalicular membrane (Clayton et al., J.Pediatr. 67:1025 (1965)) and on the fact that all previously identified proteins that conduct active (in to out) transport across this membrane are ABC-proteins (Muller et al., Am.J. Physiol. 272, G1285 (1997)).

We identified transcripts from the region by hybridizing 250,000 plated plaques from an amplified oligo dT-primed human livre cDNA library (ATCC#77402) with the entire insert from PAC 52L12 as a probe. Filters and probe that this partial cDNA exhibits strong similarity (56%) to the bovine ATPase II gene. Hybridization of this cDNA to Northern blots (Clontech) revealed a transcript of approximately 7 KB. This transcript was highly expressed in proximal small intestine and was also present in several other tissues, including liver (Figure 2). Althoug the transcript appears particularly highly expressed in the pancreas, this lane is loaded with several fold more RNA than the other lanes.

To identiy additional coding sequence from this gene, we screened an intestinal cDNA library, continued sequence scanning, performed 5' rapid amplification of cDNA ends (RACE), and screened the EST database with the additional

sequence obtained. We screened 1,350,000 phage from an amplified oligo dT- and random-primed human proximal small intestine cDNA library (Clontech), using the 1.6 KB cDNa sequence, or its most 5' end, as a probe. Nineteen cDNAs were identified and characterized. Six of these clones extended the cDNA sequence 3' (yielding 1.15 KB of new sequence), and two of them, 5' (yielding 0.46 KB of new sequence). The addition 3' sequence aligned with another partial cDNA identified in the original PAC screen. Iterative screening of the EST database identified six ESTs that contain sequence from the 3' untranslated region of the gene (Genbank accession numbers N56820/N32400 (I.M.A.G.E. Consortium CloneID No. 258798), AA257072/AA257166 (I.M.A.G.E. Consortium CloneID No. 682176), AA234928/AA234929, T10596, AA532514, D79900). Sequence scanning was performed on PAC 63L4 (Figure 1B), which has an insert of approximately 175 KB, and was shown to contain the more 5' portion of the 1.6 KB cDNA. 245 clone ends werd sequenced and identified as human, yielding 147 KB of raw human sequence which, assembled into contigs, resulted in 92 KB of non-redundant sequence. The sequence was analyzed with BLAST and, in most cases, XGRAIL (Wisconsin Package Version 9.1, Genetics Computer Grout (GCG), Madison, Wisc.). Seven "excellent" candidate exons werd identified by GRAIL 2. Two of these exons were present in the sequence obtained by cDNA library screening. One exon was novel and had strong similarity to a region of the bovine ATPase II 50 amino acids from the 5' end of the coding sequence. The intervening 1 KB of cDNA sequence between this exon and the 5'-most sequence obtained by cDNA library screening was obtained by PCR from human liver cDNA. Based on sequence homology, four "excellent" candidate exons identified did not appear to be a part of this candidate cDNA, and did not demonstrate strong

similarity to any known genes. However, one of these later proved to be the exon containing the translation start site (boundaries predicted incorrectly by GRAIL). To obtain the most 5' portion of the coding sequence, RACE was performed, using the 5' RACE system (Gibco BRL). Total human liver RNA was reverse-transcribed with a gene-specific primer. Hemi-nested PCR was then performed using two additional gene-specific primers, yielding 340 bp of additional sequence.

These approaches yielded a total of 5.9 KB of cDNA sequence. We termed the complete sequence ofthis candidate cDNA, FIC1-1 (Figure 1C). This sequence included 61 bp of 5' untranslated region (UTR), followed by the putative translation start site, and the complete 3753 bp coding sequence of FIC1-1. The stop codon is followed by 2.1 KB of 3' UTR and ends with a polyA tail. Alu repeat sequence was observed in the 5' and 3' UTRs, 40 bp and 495 bp in length, respectively.

The 3.8 KB coding sequence of FIC1-1 contains the "signature" motifs that characterize P-type ATPases, as a whole (Fagan et al., J.Mol.Evol. 38, 57 (1994); Lutsenko et al., Biochem. 34:15608 (1995)). Within these motifs in this cDNA, specific sequences are present that define a recently identified family of P-type ATPase genes that encode putative aminophospholipid transporters (Tang et al., Science 272:1495 (1996)). These sequences differentiate this family from genes for previously recognized P-type ATPases, which transport cations. When the amino acid sequence predicted for FIC1-1 was compared with publicly available sequences using BLAST (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisc.), it exhibited strong similarity (52%) to that of bovine ATPase II, the first described member of this family (Tang et al., Science

272:1495 (1996)), and to the mouse orthologue of this bovine gene (53%). The P-type ATPase family of which FIC1-1 is a member was previously known to contain four genes, the bovine ATPase II and one gene each from S.cerevisiae (drs2= Genbank accession number P39524), C.elegans (Genbank accession number U28940), and P.falciparum (Genbank accession number U16955). Through screening of publicly available databases using BLAST, we identified several additional yeast (S.cerevisiae and S.pombe) and C.elegans genes that exhibit sequences characteristic of this P-type ATPase family, and also noted the mouse orthologue of bovine ATPase II (Genbank accession number U75321).

FIC1-1 exhibited 89% similarity to an EST representing 89 amino acids of a cDNA from porcine small intestine (Genbank accession number F14675). This similarity persisted in a region where the sequence of FIC1-1 diverges substantially from that of the cDNA for bovine ATPase II. Furthermore, we subsequently identified a distinct partial cDNA from a human small intestine library with a predicted amino acid sequence that is much more similar to that of bovine ATPase II than is the amino acid sequence predicted for FIC1-1. These observations indicate that FIC1-1 is not the human counterpart of the bovine ATPase II gene.

Samples of tissue in which FIC1-1 was expressed were not available for most of the PFIC1 and BRIC patients in this study, and thus we searched for mutations in their genomic DNA. Exon-intron boundaries in FIC1-1 were identified and intronic primers were chosen to allow amplification of exons. Since PFIC demonstrated locus heterogeneity, and BRIC mas as well (Strautnieks et al., Am.J.Hum.Genet. 61:630-633 (1997), and Sinke et al., Hum.Genet. 100:382-387 (1997)), we screened for mutations in PFIC and BRIC patients who either were homozygous for many consecutive markers in the

candidate region, or carried at least one copy of an extended conserved haplotype identified in multiple patients. We also included patient S.K., who carried a deletion of part of the candidate region on one chromosome. Five PFIC1 mutations and two BRIC mutations were identified in FIC1-1, indicating that this gene is responsible for both disorders (Figure 4). In the patients in whom these mutations were identified we sequenced exons spanning the 3.8 KB coding sequence of FIC1-1; no other sequence variant were found that were not also present in controls.

In PFIC1 patients, we identified 3 missense mutations, one deletion, and one skipped exon. One missense mutation G308V (G923T), replaces a glycine that is highly conserved in P-type ATPases (Moller et al., Biochim. et Biophys, Acta 1286:1 (1996)) (Figure 4). This mutation is found on the PFIC1 haplotype identified in the Amish; all nine Amish PFIC1 patients shown to be homozygous tor the same haplotype were also homozygous for this mutation. It was not present on 107 control chromosomes.

A second missense mutation, G892R (G2674A), present in homozygous form in a patient of European descent, replaced a very highly conserved glycine present in a signature motif thought to be involved in ATP-binding in P-type ATPases (Figure 4). This mutation was not present in a sample of 82 control chromosomes. The third missense mutation, L288S (T863C), was identified in a Polish patient, and occurs in a position in which only conservative substitutions are generally seen in the family of P-type ATPases to which FIC1-1 belongs (Figure 4). It was not present on 107 control chromosomes.

We further defined the boundaries of the deletion present on one chromosome in PFIC1 patient S.K. through pulsed-field gel electrophoretic (PFGE) analysis; this

deletion includes the 5' 1.4 KB of the coding sequence of FIC1-1 (Figure 4). PFGE analysis of lymphoblastoid cell line DNA from patient S.K., her unaffected mother (also heterozygous for the deletion), and controls was performed, using probes from different regions of FIC1-1. Characterization of fragments of altered size, and detection of decreased dosage of fragments of normal size, in the deletion carriers allowed localization of the deletion breakpoint. This result is consistent with the observed orientation of the gene. To identify the mutation on the non-deleted chromosome in this patient, we screened genomic DNA. This patient was heterozygous for a mutation 2097+2(T-c) in the splice site consensus sequence immediately 3' of the exon which encodes amino acid residues 645-699 (Figure 4). This exon is predicted to code for part of the largest cytoplasmic domain. Reverse transcription (RT) PCR of this region of FIC1-1 was performed using RNA from this patient's liver .The predominant PCR product was of smaller size than that seen in controls. Sequencing of this product, which derived from the non-deleted chromosome of patient S.K., showed that this exon was skipped; skipping of this exon does not change the reading frame of FIC1-1. The PCR experiment that revealed exon skipping in patient S.K. used a primer that is situated within the 1.4 KB deletion; this indicated that the skipped exon is on this patient's non-deleted chromosome.

In BRIC patients, we identified a missense mutation and a small deletion. The missense mutation, I661T (T1982C), is a non-conservative mutation at a site where the genes in this family of P-type ATPases all encode either leucine or isoleucine (Figure 4). This mutation is present on the most common conserved haplotype in BRIC patients of Western European descent, being observed in patients from 19 families,

and was not found on 84 control chromosomes. A Dutch patient was found to be homozygous for a deletion of 3 aminoi acids, 795delGNR (2384delGAACCGTG). This mutation occurs in a region of the gene that demonstrates little conservation, except between FIC1-1 and its porcine homologue, in which the three amino acids are identical. This mutation was not found on 90 control chromosomes. This patient also displayed a homozygous sequence alteration in the intronic sequence that precedes the exon containing the deletion, 2286-4, -3(CT-AA). This alteration was not present in 90 control chromosomes. Fibroblast RNA was available from this patient. When the portion of FIC1-1 that contains this exon was amplified using RT-PCR, the predominant product was of expected size. However, a less abundant product of smaller sizer was also obtained (and was not observed in controles); sequencing of this smaller product showed that the exon containing the deletion was skipped. It is not certain whether this exon skipping contributes to the phenotype, or if the disease is due to the deletion alone.

Normal controls used to evaluate the possible PFIC1 and BRIC mutations were from the following sources: 1) For PFIC1 mutations present in Amish and Polish patients; 29 Amish, 36 Polish, and 42 other European chromosomes; 2) For the G892R PFIC1 mutation: 82 European chromosomes, 3) For the I661T BRIc mutation: 84 European chromosomes; 4) For the 795delGNR, 2286-4, -3(CT-AA) BRIC mutation: 90 European chromosomes.

We term the protein transcribed from the FIC1 gene FIC1. FIC1 is the first identified human member of a recently reported family of P-type ATPases whose other members are believed to transport aminophospholipids (Tang et al., Science 272:1495 (1996)). The fact that mutations in this gene are responsible for two forms of cholestasis, and that

the closely related bovine ATPase II plays an essential role in several different components of reticuloendothelial system function (Tang et al., Science 272:1495 (1996)), suggests that this new family is important in the physiology of a wide range of organ systems in mammals. The PFIC1 mutations that we have identified are likely to destroy the function of FIC1 or to compromise it severely. The BRIC mutations identified thus far are in less highly conserved regions of the gene, suggesting that these changes may affect the function of FIC1 less drastically than do PFIC1 mutations.

FIC1 presumably plays a role in the enterohepatic circulation of bile acids, which consists of three broad steps. Bile acids are synthesized in hepatocytes. They are secreted across the canalicular membrane into the biliary tract and thence into the small intestine. In the intestine they are absorbed and returned, through the portal circulation, to the liver where they are re-secreted into bile (F.Hofman, Gastointestinal and Liver Disease, M.Feldman M.H.Sleisenger, and B.F.Scharschmidt, Eds. (WB Saunders Co., Philadelphia, ed. 6, 1997), pp.937-947). As bile acid synthesis is not defective in BRIC or PFIC1 (Bijleveld et al. Hepatology 9:532 (1989); Bijleveld et al., Gastroenterology 97:427 (1989); Bull et al., Hepatology 25:155 (1997)), FIC1 probably plays a role in transport of bile acids to the canaliculus, uptake of bile acids from intestinal contents into intestinal mucosa, or both.

Defective canalicular transport would be expected to result in liver injury through intrahepatic accumulation of bile acids. Patients with both disorders display concentrations of bile acids which are elevated in serum but diminished in bile (Bull et al., Hepatology 26:155 (1997); Tazawa et al., J.Pediatr.Gastroenterol.Nutr. 4:32 (1985)).

Additionally, PFIC1 patients experience dramatic amelioration of cholestasis following liver transplant (Knisely, J. Pediatr.Gastroenterol.Nutr. 21:328 (1995)). It is conceivable that FIC1 directly transports bile acids (which are, like phospholipids, amphipathic organic molecules) into bile, however, all previously identified molecules that mediate active in-to-out transport across the canalicular membrane are members of the ATP-binding cassette (ABC) protein family (Muller et al., Am.J.Physiol. 272:G1285 (1997)).

One might hypothesize that FIC1 plays a role in phospholipid transport at the canalicular membrane. Under normal circumstances phospholipids at this membrane, as at other membranes in eukaryotic cells, are likely symmetrically distributed, with phosphatidylcholine concentrated in the outer leaflet and aminophospholipids concentrated in the inner leaflet (Williamson et al., Mol.Membr.Biol. 11:199 (1994); Zhao et al., Biochim.Biophys.Acta 1357:57 (1997)). Transport of phosphatidylcholine from the hepatocyte across the canalicular membrane, which is essential for bile formation, is almost certainly mediated by a previously identified ABC-protein (Smit et al., Cell 75:451 (1993); Reutz et al., Cell 77:1071 91994); van Helvoort et al., Cell 87:507 (1996)). FIC1's function could be consistent with that proposed for other members of its family of P-type ATPases, if it transported aminophospholipids from the outer to the inner leaflet of the canalicular membrane, thus maintaining the asymmetric phospholipid distribution that is important for proper membrane function (Tang et al., Science 272:1495 (1996). Under this scenario, FIC1 would facilitate rather than directly mediate canalicular bile acid transport.

The finding that FIC1 is expressed at particularly

high levels in small intestine is consistent with several lines of evidence suggesting that intestinal processing of bile acids is abnormal in PFIC1 and BRIC. A defect in transport of either bile acids or phospholipids, imparing bile acid reabsorption in intestine, could account for the striking malabsorption and diarrhea in patients with PFIC1 (Clayton et al., J.Pediatr. 67:1025 (1965); Linarelli et al. J.Pediatr. 81:484 (1972)). This diarrhea does not resolve in PFIC1 patients following liver transplants, a finding that distinguishes these patients from individuals with other forms of PFIC (Knisely, J.Pediatr.Gastroenterol.Nutr. 21:328 (1995); Knisely et al., Arch.Dis.Child. 77:276 (1997)), for whom liver transplants are essentially curative. Malabsorption of bile acids in the small intestine could also account for the excessive fecal bile acid loss described in some patients with BRIC (Bijleveld et al. Hepatology 9:532 (1989), Bijleveld et al., Gastroenterology 97:427 (1989)) as well as the increased conversion by enteric flora of chenodeoxycholic acid to lithocholic acid, a bile acid which is known to cause experimental cholestasis and which is increased in the blood and bile of patients with BRIC and PFIC1, but not in samples of PFIC patients of indeterminate type (Bijleveld et al. Hepatology 9:532 (1989); Bijleveld et al., Gastroenterology 97:427 (1989), Linarelli et al., J.Pediatr. 81:484 (1972); Jacquemin et al., Eur.J. Pediatr. 153:424 (1994)). The clinical consequences of such intestinal bile acid malabsorption would be expected to vary in relation to factors such as diet, infection, gastrointestinal motility, and the composition of enteric flora. Such factors (which could, for example, alter the level of lithocholate production) could explain the strikingly variabel and episodic course of BRIC, as well as the observation that episodes frequently follow viral

illnesses (Bijleveld et al., Hepatology 9:532 (1989); Bijleveld et al., Gastroenterology 97:427 (1989)).

Although the data discussed above support the hypothesis that FIC1 is important in both the intestinal and hepatic phases of bile acid circulation, other evidence suggests an even wider role for this protein. A complete model for FIC1's function accounts for observations in obligate carriers of PFIC1 mutations that suggest generalized abnormalities in organic anion handling by the liver (Linarelli et al., J.Pediatr. 81:484 (1972)), as well as the finding that sweat electrolyte concentrations are frequently abnormal in PFIC1 patients (Knisely et al., Arch.Dis.Child. 77:276 (1997); Bourke et al., Arch.Dis. Child. 75:223 (1996); Lloyd-Still, J.Pediatr. 99:580 (1981)). These facts, together with the observation that FIC1 is expressed in a wide variety of epithelial tissues (including sites not known to be involved in bile acid handling), suggest that this protein may prove to act globally at sites of secretion and absorption.

All references cited herein are expressly incorporated in their entirety for all purposes.

CLAIMS

1. An isolated and/or recombinant nucleic acid, or a functional fragment or homologue thereof, derived from a gene, which gene comprises a mutation in patients with benign recurrent intrahepatic cholestasis (BRIC) or progressive familial intrahepatic cholestasis (PRIC), said nucleic acid in humans having a sequence substantially idential to a nucleic acid sequence as shown in Fig.5.

2. A nucleic acid according to claim 1 of human origin.

3. A nucleic acid according to claim 1 or 2 encoding a protein or functional fragment thereof having P-type ATPase-like activity.

4. A vector comprising a nucleic acid according to anyone of claims 1 to 3.

5. A cell comprising a nucleic acid according to anyone of claims 1 to 3 or a vector according to claim 4.

6. A transgenic animal comprising a nucleic acid according to anyone of claims 1 to 3 or a vector according to claim 4 or a cell according to claim 5.

7. An isolated protein or functional fragment thereof encoded by a nucleic acid derived from a gene which, in humans, comprises a mutation in patients with benign recurrent intrahepatic cholestasis (BRIC) or progressive familial intrahepatic cholestasis (PFIC), said protein having in humans an amino acidsequence substantially identical to a sequence as shown in figure 3.

8. A protein or functional fragment thereof expressed from a vector according to claim 4 or a cell according to claim 5.

9. An antibody specifically directed against a protein according to claim 7 or 8.

10. A gene delivery vehicle comprising a nucleic acid

according to anyone of claims 1 to 3, or a vector according to claim 4.

11. Use of a nucleic acid according to anyone of claims 1 to 3, or a vector according to claim 4, or a cell according to claim 5, or an animal according to claim 6, or a protein according to claim 7 or 8 or an antibody according to claim 9, or a gene delivery vehicle according to claim 10 for the development and/or detection of a drug for the treatment of a disorder related to membrane malfunction.

12. Use according to claim 11 wherein said disorder is related to cholestasis.

13. A pharmaceutical composition comprising a nucleic acid according to anyone of claims 1 to 3, or a vector according to claim 4, or a cell according to claim 5, or an animal according to claim 6, or a protein according to claim 7 or 8 or an antibody according to claim 9, or a gene delivery vehicle according to claim 10.

14. A composition according to claim 13 for the treatment of cholestasis or disorders related to membrane malfunctioning such as for example BRIC or PFIC or intestinal disorders.

15. Use of a nucleic acid according to anyone of claims 1 to 3, or a vector according to claim 4, or a cell according to claim 5, or an animal according to claim 6, or a protein according to claim 7 or 8 or an antibody according to claim 9, or a gene delivery vehicle according to claim 10 in the preparation of a medicament for the treatment of cholestasis or disorders related to membrane malfunctioning, such as for example BRIC or PFIC or intestinal disorders.

16. A method of treating cholestasis in a patient, the method comprising administering to the patient a therapeutic dose of a composition according to calim 13 or 14.

17. The method of claim 16 wherein the cholestasis is benign recurrent intrahepatic cholestasis (BRIC).

18. The method of claim 16 wherein the cholestasis is progressive familial intrahepatic cholestasis type 1 (PFIC1).

19. A method for detecting a mutation in the FIC1 gene, the mehtod comprising amplifying a FIC1 gene sequence in a nucleotide sample from a patient using a primer substnatially idential to a nucleotide sequence of at least 8 consecutive nucleotides of Figure 5.

20. The method of claim 19 wherein the mutation results in cholestasis in a patient.

21. The method of claim 19, wherein the mutation is G308V, G892R, L288S, 2097+2(T-C), I1661T, 795delGNR, 2286-4, -3(CT-AA).

22. A method for diagnosing the disease cholestasis in a patient, the method comprising detecting a mutation in the FIC1 gene, wherein the presence of the mutation indicates a likelihood of having cholestasis.

23. The method of claim 22, wherein the mutation is G308V, G892R, L288S, 2097+2(T-C), I1661T, 795delGNR, 2285-4, -3(CT-AA).

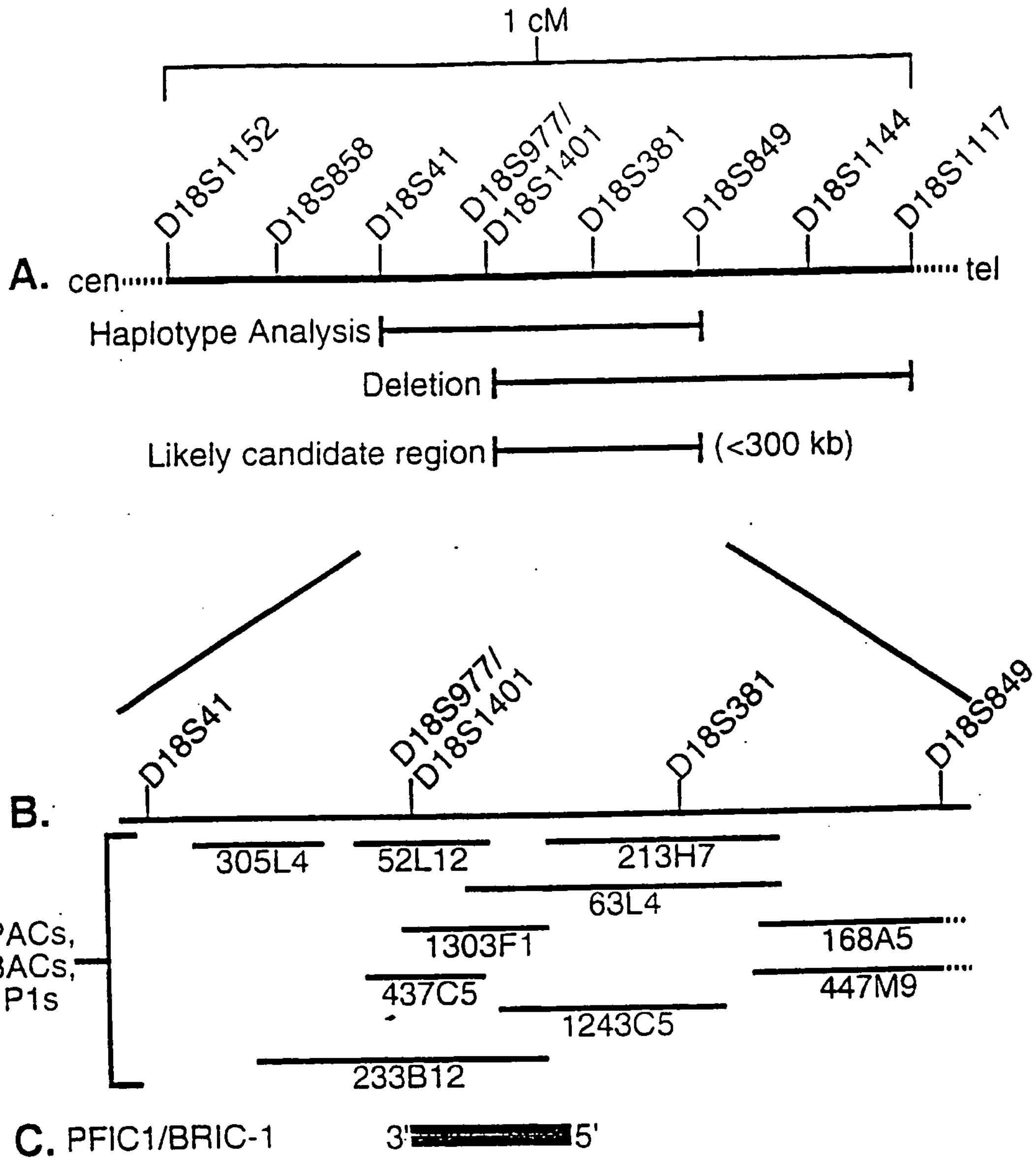
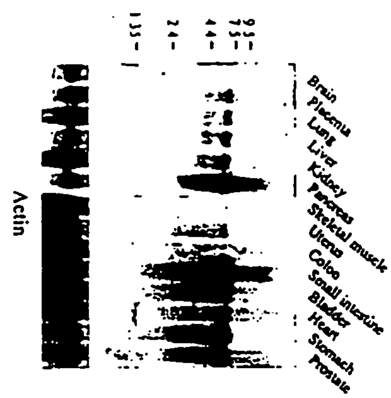

1 cM
D18S1152
D18S858
D18S41
D18S977/
D18S1401
D18S381
D18S849
D18S1144
D18S1117
A. cen
tel
Haplotype Analysis
Deletion
Likely candidate region
(<300 kb)
D18S41
D18S977/
D18S1401
D18S381
D18S849
B.
PACs,
BACs,
P1s
305L4
52L12
213H7
63L4
1303F1
168A5
437C5
447M9
1243C5
233B12
C. PFIC1/BRIC-1
3'
5'

Figure 1

Figure 2


Actin
Brain
Placenta
Lung
Liver
Kidney
Pancreas
Skeletal muscle
Uterus
Colon
Small intestine
Bladder
Heart
Stomach
Prostate

1 MSTERDSETT FDEDSQPNDE VVPYSDDETE DELDDQGSAV EPEQNRVNRE AEENREPFRK ECTWQVKAND

71 RKYHEQPHFM NTKFLCIKES KYANNAIKTY KYNAFTFIPM NLFEQFKRAA NLYFLALLIL QAVPQISTLA

141 WYTTLVPLLV VLGVTAIKDL VDDVARHKMD KEINNRTCEV IKDGRFKVAK WKEIQVGDVI RLKKNDFVPA

211 DILLLSSSEP NSLCYVETAE LDGETNLKFK MSLEITDQYL QREDTLATFD GFIECEEPNN RLDKFTGTLF

281 WRNTSFPLDA DKILLRGCVI RNTDFCHGLV IFAGADTKIM KNSGKTRFKR TKIDYLMNYM VYTIFVVLIL

351 LSAGLAIGHA YWEAQVGNSS WYLYDGEDDT PSYRGFLIFW GYIIVLNTMV PISLYVSVEV IRLGQSHFIN

421 WDLQMYYAEK DTPAKARTTT LNEQLGQIHY IFSDKTGTLT QNIMTFKKCC INGQIYGDHR DASQHNHNKI

491 EQVDFSWNTY ADGKLAFYDH YLIEQIQSGK EPEVRQFFFL LAVCHTVMVD RTDGQLNYQA ASPDEGALVN

561 AARNFGFAFL ARTQNTITIS ELGTERTYNV LAILDFNSDR KRMSIIVRTP EGNIKLYCKG ADTVIYERLH

631 RMNPTKQETQ DALDIFANET LRTLCLCYKE IEEKEFTEWN KKFMAASVAS TNRDEALDKV YEEIEKDLIL

701 LGATAIEDKL QDGVPETISK LAKADIKIWV LTGDKKETAE NIGFACELLT EDTTICYGED INSLLHARME

771 NQRNRGGVYA KFAPPVQESF FPPGGNRALI ITGSWLNEIL LEKKTKRNKI LKLKFPRTEE ERRMRTQSKR

841 RLEAKKEQRQ KNFVDLACEC SAVICCRVTP KQKAMVVDLV KRYKKAITLA IGDGANDVNMIKTAHIGVGI

911 SGQEGMQAVM SSDYSFAQFR YLQRLLLVHG RWSYIRMCKF LRYFFYKNFA FTLVHFWYSF FNGYSAQTAY

981 EDWFITLYNV LYTSLPVLLM GLLDQDVSDK LSLRFPGLYI VGQRDLLFNY KRFFVSLLHG VLTSMILFFI

1051 PLGAYLQTVG QDGEAPSDYQ SFAVTIASAL VITVNFQIGL DTSYWTFVNA FSIFGSIALY FGIMFDFHSA

1121 GIHVLFPSAF QFTGTASNAL RQPYIWLTII LTVAVCLLPV VAIRFLSMTI WPSESDKIQK HRKRLKAEEQ

1191 WQRRQQVFRR GVSTRRSAYA FSHQRGYADL ISSGRSIRKK RSPLDAIVAD GTAEYRRTGD S

Figure 3

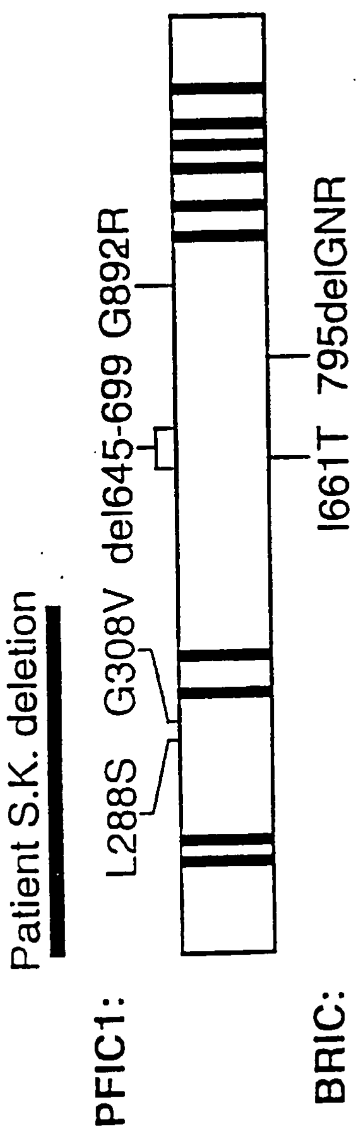

PFIC1:
Patient S.K. deletion
L288S
G308V
del645-699
G892R
BRIC:
I661T
795delGNR

Figure 4

[Strand]

1 ATTTTCCTGCCTCAGCCTCC CGAGCAGCTTGGACTACAGG TAGTTCCAATTTGCCAGCAG
61 AATGAGTACAGAAAGAGACT CAGAAACGACATTTGACGAG GATTCTCAGCCTAATGACGA
121 AGTGGTTCCCTACAGTGATG ATGAAACAGAAGATGAACTT GATGACCAGGGGTCTGCTGT
181 TGAACCAGAACAAAACCGAG TCAACAGGGAAGCAGAGGAG AACCGGGAGCCATTCAGAAA
241 AGAATGTACATGGCAAGTCA AAGCAAACGATCGCAAGTAC CACGAACAACCTCACTTTAT
301 GAACACAAAATTCTTGTGTA TTAAGGAGAGTAAATATGCG AATAATGCAATTAAAACATA
361 CAAGTACAACGCATTTACCT TTATACCAATGAATCTGTTT GAGCAGTTTAAGAGAGCAGC
421 CAATTTATATTTCCTGGCTC TTCTTATCTTACAGGCAGTT CCTCAAATCTCTACCCTGGC
481 TTGGTACACCACACTAGTGC CCCTGCTTGTGGTGCTGGGC GTCACTGCAATCAAAGACCT
541 GGTGGACGATGTGGCTCGCC ATAAAATGGATAAGGAAATC AACAATAGGACGTGTGAAGT
601 CATTAAGGATGGCAGGTTCA AAGTTGCTAAGTGGAAAGAA ATTCAAGTTGGAGACGTCAT
661 TCGTCTGAAAAAAAATGATT TTGTTCCAGCTGACATTCTC CTGCTGTCTAGCTCTGAGCC
721 TAACAGCCTCTGCTATGTGG AAACAGCAGAACTGGACGGA GAAACCAATTTAAAATTTAA
781 GATGTCACTTGAAATCACAG ACCAGTACCTCCAAAGAGAA GATACATTGGCTACATTTGA
841 TGGTTTTATTGAATGTGAAG AACCCAATAACCGACTAGAT AAGTTTACAGGAACACTATT
901 TTGGAGAAACACAAGTTTTC CTTTGGATGCTGATAAAATT TTGTTACGTGGCTGTGTAAT
961 TAGGAACACCGATTTCTGCC ACGGCTTAGTCATTTTTGCA GGTGCTGACACTAAAATAAT
1021 GAAGAATAGTGGGAAAACCA GATTTAAAAGAACTAAAATT GATTACTTGATGAACTACAT
1081 GGTTTACACGATCTTTGTTG TTCTTATTCTGCTTTCTGCT GGTCTTGCCATCGGCCATGC
1141 TTATTGGGAAGCACAGGTGG GCAATTCCTCTTGGTACCTC TATGATGGAGAAGACGATAC
1201 ACCCTCCTACCGTGGATTCC TCATTTTCTGGGGCTATATC ATTGTTCTCAACACCATGGT
1261 ACCCATCTCTCTCTATGTCA GCGTGGAAGTGATTCGTCTT GGACAGAGTCACTTCATCAA
1321 CTGGGACCTGCAAATGTACT ATGCTGAGAAGGACACACCC GCAAAAGCTAGAACCACCAC
1381 ACTCAATGAACAGCTCGGGC AGATCCATTATATCTTCTCT GATAAGACGGGGACACTCAC
1441 ACAAAATATCATGACCTTTA AAAAGTGCTGTATCAACGGG CAGATATATGGGGACCATCG
1501 GGATGCCTCTCAACACAACC ACAACAAAATAGAGCAAGTT GATTTTAGCTGGAATACATA
1561 TGCTGATGGGAAGCTTGCAT TTTATGACCACTATCTTATT GAGCAAATCCAGTCAGGGAA
1621 AGAGCCAGAAGTACGACAGT TCTTCTTCTTGCTCGCAGTT TGCCACACAGTCATGGTGGA
1681 TAGGACTGATGGTCAGCTCA ACTACCAGGCAGCCTCTCCC GATGAAGGTGCCCTGGTAAA
1741 CGCTGCCAGGAACTTTGGCT TTGCCTTCCTCGCCAGGACC CAGAACACCATCACCATCAG
1801 TGAACTGGGCACTGAAAGGA CTTACAATGTTCTTGCCATT TTGGACTTCAACAGTGACCG
1861 GAAGCGAATGTCTATCATTG TAAGAACCCCAGAAGGCAAT ATCAAGCTTTACTGTAAAGG
1921 TGCTGACACTGTTATTTATG AACGGTTACATCGAATGAAT CCTACTAAGCAAGAAACACA
1981 GGATGCCCTGGATATCTTTG CAAATGAAACTCTTAGAACC CTATGCCTTTGCTACAAGGA
2041 AATTGAAGAAAAAGAATTTA CAGAATGGAATAAAAAGTTT ATGGCTGCCAGTGTGGCCTC
2101 CACCAACCGGGACGAAGCTC TGGATAAAGTATATGAGGAG ATTGAAAAAGACTTAATTCT
2161 CCTGGGAGCTACAGCTATTG AAGACAAGCTACAGGATGGA GTTCCAGAAACCATTTCAAA
2221 ACTTGCAAAAGCTGACATTA AGATCTGGGTGCTTACTGGA GACAAAAAGGAAACTGCTGA
2281 AAATATAGGATTTGCTTGTG AACTTCTGACTGAAGACACC ACCATCTGCTATGGGGAGGA
2341 TATTAATTCTCTTCTTCATG CAAGGATGGAAAACCAGAGG AATAGAGGTGGCGTCTACGC

Figure 5

[Strand]

2401 AAAGTTTGCACCTCCTGTGC AGGAATCTTTTTTTCCACCC GGTGGAAACCGTGCCTTAAT
2461 CATCACTGGTTCTTGGTTGA ATGAAATTCTTCTCGAGAAA AAGACCAAGAGAAATAAGAT
2521 TCTGAAGCTGAAGTTCCCAA GAACAGAAGAAGAAAGACGG ATGCGGACCCAAAGTAAAAG
2581 GAGGCTAGAAGCTAAGAAAG AGCAGCGGCAGAAAAACTTT GTGGACCTGGCCTGCGAGTG
2641 CAGCGCAGTCATCTGCTGCC GCGTCACCCCCAAGCAGAAG GCCATGGTGGTGGACCTGGT
2701 GAAGAGGTACAAGAAAGCCA TCACGCTGGCCATCGGAGAT GGGGCCAATGACGTGAACAT
2761 GATCAAAACTGCCCACATTG GCGTTGGAATAAGTGGACAA GAAGGAATGCAAGCTGTCAT
2821 GTCGAGTGACTATTCCTTTG CTCAGTTCCGATATCTGCAG AGGCTACTGCTGGTGCATGG
2881 CCGATGGTCTTACATAAGGA TGTGCAAGTTCCTACGATAC TTCTTTTACAAAAACTTTGC
2941 CTTTACTTTGGTTCATTTCT GGTACTCCTTCTTCAATGGC TACTCTGCGCAGACTGCATA
3001 CGAGGATTGGTTCATCACCC TCTACAACGTGCTGTACACC AGCCTGCCCGTGCTCCTCAT
3061 GGGGCTGCTCGACCAGGATG TGAGTGACAAACTGAGCCTC CGATTCCCTGGGTTATACAT
3121 AGTGGACAAAGAGACTTAC TATTCAACTATAAGAGATTC TTTGTAAGCTTGTTGCATGG
3181 GGTCCTAACATCGATGATCC TCTTCTTCATACCTCTTGGA GCTTATCTGCAAACCGTAGG
3241 GCAGGATGGAGAGGCACCTT CCGACTACCAGTCTTTTGCC GTCACCATTGCCTCTGCTCT
3301 TGTAATAACAGTCAATTTCC AGATTGGCTTGGATACTTCT TATTGGACTTTTGTGAATGC
3361 TTTTTCAATTTTTGGAAGCA TTGCACTTTATTTTGGCATC ATGTTTGACTTTCATAGTGC
3421 TGGAATACATGTTCTCTTTC CATCTGCATTTCAATTTACA GGCACAGCTTCAAACGCTCT
3481 GAGACAGCCATACATTTGGT TAACTATCATCCTGACTGTT GCTGTGTGCTTACTACCCGT
3541 CGTTGCCATTCGATTCCTGT CAATGACCATCTGGCCATCA GAAAGTGATAAGATCCAGAA
3601 GCATCGCAAGCGGTTGAAGG CGGAGGAGCAGTGGCAGCGA CGGCAGCAGGTGTTCCGCCG
3661 GGGCGTGTCAACGCGGCGCT CGGCCTACGCCTTCTCGCAC CAGCGGGGCTACGCGGACCT
3721 CATCTCCTCCGGGCGCAGCA TCCGCAAGAAGCGCTCGCCG CTTGATGCCATCGTGGCGGA
3781 TGGCACCGCGGAGTACAGGC GCACCGGGGACAGCTGATCC CTTACCCCCAGGCTGGGATG
3841 CGGCCACAAAGCACGTCTAT TTTTTTATGAAAGACTCTCA GGACTTTCTGTGTGTGTGTG
3901 AATTGCATTCATCACAAAGA TATTGAAGACAATAAATAAT CTTTATAACAAACTCCTTGG
3961 GTTGGACTATTAAACAATAG CAGAGCCAAGAAATAGGACT TTTAAAAGTCCTATTATTCG
4021 GCCGGGCGTGGTGGCTCAGG TCTGTAATCCCAGCACTTTA GGAGGCTGAGGAGGGGAGAT
4081 CACCTGAGGCCAGGAGTTCG AGACCAGCCCAGCCAACATG GTGAAACCCTGTCTCTACCA
4141 TAAATTAAAAATTAGCCGGG CTTGGTGGTGGGCACCCGTA ATCCCACCTATTTGAGAAGC
4201 TGAAGCAGAAGAATCGCTTG AACCTGGGAGATGGAGGTTA CAGTGAGCCGAGATTGCGCC
4261 ACTGCACTCCAGCCTGGGTG ACAGAGCAAGACTCTGTCTT AAGAAAAATTTAAAAATAGG
4321 CCGGGCATGGTGGCTCATGC CTGTCATCCTAGCACTTTGG GAGGCCAAGGCAGGTGGATC
4381 ACTTGAGGTCAGGAGACCAG CCCGGCCAACATGGTGAAAC CCCATCTCTACTAAAAATAC
4441 AAAAATTAGCTGGAAATCGC TTGAAACCTGGGAGGTGGAG GTTCCAGTGGAGCCGAGATC
4501 GTGGCACTGCACTCCCAACC TGAGCAACAGAGTGAGACAC CAGCTCMAAAAAAAATTTTT
4561 TTAATAATAATAAAAGTCCT ATTATTCAACTGGTTATGTA CATTATGGTTGAAAGGGAAC
4621 GTTTTAATCCCAGTCTCAAT CCAGGGCAATAGAATTACAA AGCATGTTGTATTTCAGTTC
4681 AAATGGTATTGTATTATAAA ATTACAGTTACATTTTCCTT TCGGTGATCTTCAGCATAAT
4741 TTCCCAGAGGCCCCCTTTTTC CTCCCTATAGGCCATCTTAT TAACAGATTTTAAAATTTAT

Figure 5
cont.

[Strand]

4801 AGTAATGACAAATGACTTAT CAGTGTTCATCATCTGAAAG CTAAGTGGTTCGTTCAATCA
4861 CTTTTTCAAAGTTGATAGTA GATTGCATGGTTTCATGTTT CCTCATATTGGTTTATTAAT
4921 TCTATTTAATCAAGGAAAAT AACTTCAGATTCCATAAAGT TTCAGTTTATTTTTAGTTTA
4981 CTACTAGGTGAGATAGCACA TTACATACTTTTACTATCAA ATATTATTTTAGCAGCTTCC
5041 CATAGTACCAAATGATTTGA TTCCCTACTCTCATTTTTTA AAGCATATAAATATTTATGG
5101 GCTTAAAAAGGGGGTTTTTA AAAACTGAGGATATCAGTAA TAAATTGCAGAATATTTTGC
5161 AARGCTTTCTTTTTGGAAAG CCAAACTTTTGTGCYTGCCT ATATGCAAAGTATTTTATCA
5221 GGGACTTGAMCAARGACCTC AYTCTTTTTCACTTTGTCTT ATGTCGAGAGAAAAGGTTAT
5281 TGGCAGCCACATTCCTARGA CTTGGGGAATGGTGTGTCCT TTTAAATTTGAAGATGACTT
5341 TAGGTAATTATGGAAACTCC TCAAAGAGGAGAAAGTAATT TTTTTCCAGACATTTTTCTC
5401 ATTCTGTGTCTTTCACACAC TAGTTTCCATAGTTCGAGAA TTCTGTTTTTTACCATTGGG
5461 CTGTGAATGTTCACAATATC AGTCCTGTTGAATTCCTATG AGGTAATCACAATGTGTATA
5521 TGTTCATTTTCTAGGTATGA TAAAAGAATGTATGGCTTTT TATTCTGTGGAAGTAAAATC
5581 CTGAACGTTTACAACTTTTC CTTAACTTGTAAATAAAAAA TTGTAAGTTTTTTCTTTTTT
5641 TACAGAAAACTTAGCTTGTG TAATTCTGTTAGTTTCAGAT TTCTCTCCTGTTTTTGCAAA
5701 TTGTGGAAAGATTGACAAT GCAAATGTGTCAAAGACATA CTGTTGGGTGCAATATTAAC
5761 AATTTTAAATGCAAATTTCT TTGGATAAATTATTTCTATA TTCTGTAAATCTGAGATTTA
5821 ATGTATATTTTGTTTAAAAA AATGATTTAGTAAAATCTTT GAAAGTAAAAAAAAAAAAAA
5881 AAAAAA

Figure 5
cont.

R = A or G
Y = C or T
K = G or T
M = A or C
S = C or G
W = A or T

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